NEUROPROTECTIVE POTENTIAL OF THE N-TERMINAL BETA AMYLOID PEPTIDE FRAGMENT IN THE NEURODEGENERATION, SYNAPTIC DYSFUNCTION AND MEMORY DEFICITS IN MODELS OF ALZHEIMER'S DISEASE

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DEDICATION

Dedicated to my hero, Mama Rahma.

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ABSTRACT

Beta amyloid (Aβ) plays a central role in the pathogenesis of Alzheimer's disease (AD). It is produced by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases. This amyloidogenic pathway produces peptides 38-42 amino acids in length, based on the sites cleaved by γ-secretase. $A\beta_{1-42}$ is the predominant peptide species found in neuritic plaques. Its accumulation and impaired clearance are associated with disease progression. An alternative pathway has been proposed wherein short, N-terminal Aβ fragments are produced. The N-terminal fragments are hydrophilic, making them soluble and less likely to aggregate into plaques. $A\beta_{1-15}$ is the N-terminal Aβ fragment of focus in this study. It is produced by the sequential cleavage of APP by β- and α-secretases followed by a carboxypeptidase. $A\beta_{1-15}$ is also upregulated in AD patients suggesting a possible compensatory switch between pathways to suppress $A\beta_{1-42}$ production. We have recently shown that $A\beta_{1-15}$ can enhance long-term potentiation (LTP) in wild-type hippocampal synapses at very low (fM) concentrations. $A\beta_{1-15}$ was also shown to reverse the LTP block incurred by $A\beta_{1-42}$, as well as rescue LTP deficits in APPswe mice. This fragment has a potent and effective signaling activity via nicotinic acetylcholine receptors (nAChRs). Taken together, these data support a neuromodulatory function and a possible neuroprotective action for $A\beta_{1-15}$. This study focuses on the effects of $A\beta_{1-15}$ on $A\beta_{1-42}$ toxicity in various neuronal models, including *in vitro* NG108-15 hybrid neuroblastoma cells, *ex vivo* hippocampal neuron cultures and *in vivo* wild-type and AD model-APP mice.

We explored $A\beta_{1-15}$ neuroprotection against Aβ-mediated neurodegeneration *in vitro* by incubating our differentiated neuroblastoma cell line with different treatment combinations and across different time points in order to examine both the potency of $A\beta_{1-15}$ as well as the different ways by which $A\beta_{1-15}$ might be affecting $A\beta_{1-42}$ toxicity. Specifically, we explored the possibilities of $A\beta_{1-15}$ priming by pretreating cells with $A\beta_{1-15}$ before the addition of the toxic $A\beta_{1-42,}$ competition of the two fragments by combination treatment, or rescue experiments by adding $A\beta_{1-15}$ after addition of $A\beta_{1-42}$ to determine whether $A\beta_{1-15}$ can reverse or halt toxicity caused by $A\beta_{1-42}$. Cellular toxicity was assessed as oxidative stress (production of reactive oxygen species, ROS) and apoptotic cell death. Next, we investigated the neuroprotective effects of $A\beta_{1-15}$ *ex-vivo* by establishing primary hippocampal neuron cultures to confirm neuroprotection in a more physiologically relevant neuronal model. We then explored the potential for the $A\beta_{1-15}$ to protect or reverse (rescue) synaptic dysfunction and memory deficits resulting from Aβ synaptotoxicity. Changes in synaptic plasticity were assessed by measuring LTP in acute mouse hippocampal slices. Furthermore, we inspected the potential for rescue by $A\beta_{1-15}$ of LTP in APPswe hippocampal slices known to have LTP deficits. Lastly, we explored the effect of $A\beta_{1-15}$ in different behavior paradigms on 5XFAD (FAD: familial Alzeimer's disease) mice, a model expressing APPswe, APP-London and APP-Florida mutant transgenes as well as two mutant presenilin (PS1) transgenes, which accumulates high levels of Aβ over an accelerated timeframe (months). The behaviors examined were those related to deficits observed AD, namely contextual fear

conditioning, novel object recognition and elevated plus maze to examine effects on memory processing, recognition memory and anxiety.

We were able to show that the $A\beta_{1-15}$ protected against all measures of Aβ-triggered neurotoxicity and neuronal dysfunction: oxidative stress, DNA fragmentation, apoptotic cell death, synaptotoxicity and behavioral deficits. Notably, $A\beta_{1-15}$ prevented LTP inhibition caused by $A\beta_{1-42}$ treatment and, when injected into the hippocampus was able to rescue memory in contextual fear conditioning as well as decrease anxiety in the 5XFAD mice. To address preliminarily the possible molecular mechanisms underlying the rescue by $A\beta_{1-15}$ of memory deficits in the 5XFAD, we explored signaling pathways known to be involved in Aβ synaptotoxicity. We observed a substantial upregulation of the glutamate receptor GluR2 and phosphorylated CREB in mouse hippocampi injected with the $A\beta_{1-15}$, giving us insight into the specific actions of N-terminal fragment.

In summary, the data show that $A\beta_{1-15}$ fully protected against $A\beta_{1-42}$ –induced cellular toxicity, synaptotoxicity and behavioral deficits. Taken together, the data support our hypothesis that the N-terminal fragment ($A\beta_{1-15}$) is not only neuroprotective against acute $A\beta_{1-42}$ toxicity, but also has the ability to rescue memory in 5XFAD mice, potentially introducing a new avenue for AD therapeutics.

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LIST OF ABBREVIATIONS

Aβ-Beta Amyloid,

AD- Alzheimer's Disease

FAD- Familial Alzheimer's Disease

APP- Amyloid Precursor Protein

BACE- Beta site cleaving enzyme

nAChR- nicotinic acetylcholine receptor

ROS- reactive oxygen species

LTP- long-term potentiation

APLP- amyloid precursor-like protein,

sAPP- soluble amyloid precursor protein,

CTF- carboxy-terminal fragment,

AICD- APP intracellular domain,

CSF- cerebrospinal fluid,

aCSF- artificial cerebrospinal fluid,

WT- wild type,

NMDA- N-methyl-D-aspartate,

YFP- yellow fluorescent protein,

carboxy-H_2DCFDA- 5-carboxy-2',7'-dichlorodihydrofluorescein.

CHAPTER 1

Introduction

1. BACKGROUND

Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease that currently affects 5.2 million people in the United States, making it the most common form of dementia.[5] Dr. Alois Alzheimer first described AD (initially known as presenile dementia of the Alzheimer-type) in 1907, after the postmortem examination of a brain of a middle-aged insane asylum patient. The patient's behavioral symptoms at the asylum were described as progressive memory loss, confusion, personality changes, as well as the eventual loss of motor skills: all classical AD symptoms. Dr. Alzheimer described an atrophied brain that, when silver stained, showed abnormal aggregation of plaques and fibrils in the area where neurons had died.[6] We now know these to be the two pathological hallmarks of AD: beta amyloid plaques, and tau tangles, respectively.

The amyloid cascade hypothesis refers to the conjecture that amyloid accumulation leads to the pathogenesis of AD.[7] This hypothesis was developed after many studies showed that AD brains had significantly higher amounts of soluble Aβ compared to normal brains. More recently, however, there has been a large amount of evidence that alludes to Aβ being a neuromodulator.[8] At low levels, Aβ modulates neuroplasticity and memory, demonstrating an important physiological role that makes targeting Aβ for treatment of AD a more complicated issue.[9] It had also been demonstrated that processing of APP by different combinations of secretases will produce Aβ peptides of different lengths.[10] Of particular interest to our lab is the N-terminal fragment $A\beta_{1-15}$. While the $A\beta_{1-42}$ fragment has been shown to have toxic

effects at high concentrations,[11] $A\beta_{1-15}$ has not shown any signs of toxicity at the same concentrations. This leads us to investigate possible neuroprotective effects of the N-terminal fragment.

Amyloid Precursor Protein: Processing and Aβ Production

Amyloid precursor protein (APP) is a membrane spanning protein that belongs to a family encoded by three mammalian genes. [12,13] APP physiological functions are poorly defined and APP knockout mice have shown almost normal phenotypes, most likely because of compensation by amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2).[13] It has been suggested that APP plays a role in everything from transmembrane signal transduction, synaptogenesis, and cell adhesion to neuronal protein trafficking along axons.[14] A recent study has shown that increased APP tyrosine phosphorylation in AD neurons alters trafficking of APP, suggesting APP tyrosine phosphorylation can be a therapeutic target for AD, but more in-depth studies are required.[15] In contrast, APP processing has been widely studied because of its central role in the production of Aβ and the pathology of AD. APP goes through a regulated, sequential cleavage by three different

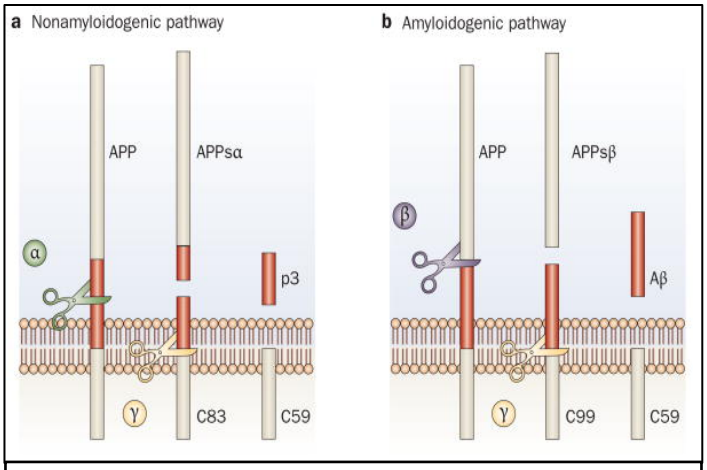

Figure 1. **APP processing pathways.**[17] a. Nonamyloidgenic pathway-APP is cleaved within the Aβ sequence by α-secrestase releasing the APPsα ectodomain followed by γ- secretase cleavage releasing the p3 fragment. b. Amyloidgenic pathway- β-secretase cleaves APP releasing APPsβ followed by γ-secretase cleavage releasing Aβ.

secretases: α-secretase, β-secretase, and γ-secretase.

There are two well established pathways by which APP is processed: the amyloidogenic pathway and the non amyloidogenic pathway (Fig.1).[16] The non amyloidegenic pathway is the predominant pathway in which APP is cleaved by α and then γ- secretases.[17,12,18] The α-secretase cleavage releases soluble APPα (sAPPα), shown to have roles in cell migration, differentiation, and adhesion.[17] The γ-secretase then cleaves the remaining carboxy-terminal fragment α (CTFα) resulting in a P3 fragment and releases an APP intracellular domain (AICD). The AICD is known to be a transcription regulator of: Neprilysin (a Aβ degrading enzyme), BACE1 (a β-secretase), and APP.[17,18]

Aβ production is achieved via the amyloidogenic pathway. Initially, β-secretase cleaves APP into sAPPβ, a fragment involved in cell viability, differentiation, axonal outgrowth and microglia activation, and a C-terminal fragment, CTFβ. The CTFβ is then cleaved by γ-secretase at multiple possible cleavage sites resulting in an Aβ fragment 38-42 amino acids in length, with the majority being $Aβ_{1-40}$ and $Aβ_{1-42}$.[17,13,19]

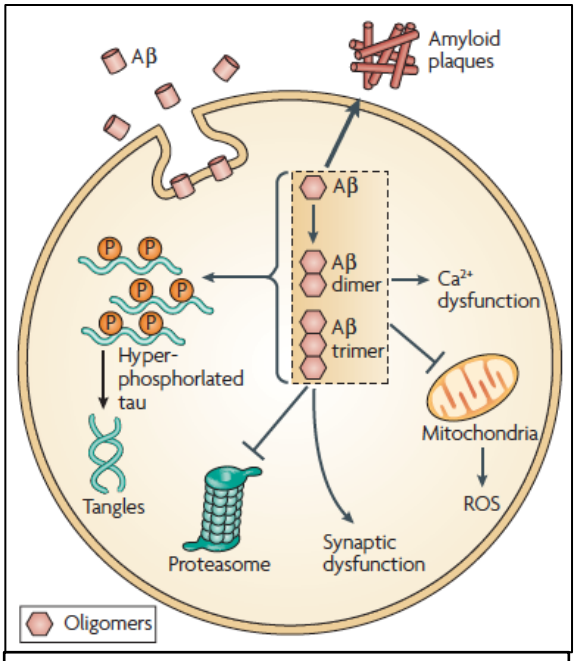

Figure 2. **Pathological effects of Aβ.** [La Ferla] Aβ aggregates into oligomers mediating pathological events such as the facilitation of tau hyper-phosphorylation, proteasome and mitochondrial disruption, calcium and synaptic dysfunction as well as contribute to ROS.

Beta Amyloid 1-42 ($A\beta_{1-42}$)

$A\beta_{1-42}$ has long been linked to AD pathology. The classic Aβ cascade hypothesis is based on the fact that Aβ aggregates into oligomeric fibrils and then into plaques. While the plaques are one of the hallmarks of AD, it is the Aβ oligomers that start a pathological cascade that leads to neurodegeneration.[20,21,22] Aβ can be generated wherever APP resides with β- and γ-secretases. Aβ generation occurs on the cell membrane and is released extracellularly, but can also occur intracellularly, in the endoplasmic reticulum, Golgi, endosomal, lysosomal, and mitochondrial membranes.[14,21,23] Aβ is predominantly released at synaptic sites.

$A\beta_{1-42}$ is more hydrophobic than $A\beta_{1-40}$ and therefore its oligomers are more likely to aggregate and cause a myriad of disruptive actions before forming the Aβ plaques seen in AD brain.[22] The overproduction of Aβ oligomers not only leads to amyloid plaques, but has also been shown to cause excitotoxicity, oxidative stress, neurotransmitter deficits, synaptic dysfunction, activation of apoptosis, and neuronal cell death (Fig.2).[21,22,24] A balance of Aβ production and clearance is imperative to avoid pathogenesis. Production and clearance of Aβ have been studied using metabolic labeling and it was apparent that clearance of Aβ in the central nervous system of AD patients was impaired.[25]

While Aβ has generally been accepted as a toxic peptide found in aged or AD brains, the discovery of soluble Aβ at very low concentrations in normal brain suggests that it has a physiological modulatory function.[14,9,26,27] While nanomolar levels of Aβ block LTP, picomolar levels enhance synaptic plasticity in electrophysiological

experiments, and memory in Morris water maze and contextual fear conditioning experiments.[9,7,27] Another study demonstrated similar results with low doses of Aβ enhancing LTP and memory in both T-maze foot shock avoidance as well as object recognition.[26] At low picomolar levels, Aβ was shown to be necessary for normal LTP and memory, as blocking $A\beta_{1-42}$ blocked LTP and caused learning deficits.[8,27] Taken together, this makes targeting of Aβ for AD treatment a complicated endeavor and alternate treatment approaches are needed.

Beta amyloid 1-15 ($A\beta_{1-15}$)

Recently, an alternative pathway was identified in which $A\beta_{1-15}$ is produced. This alternative pathway involves sequential cleavage of APP by β- and α-secretases

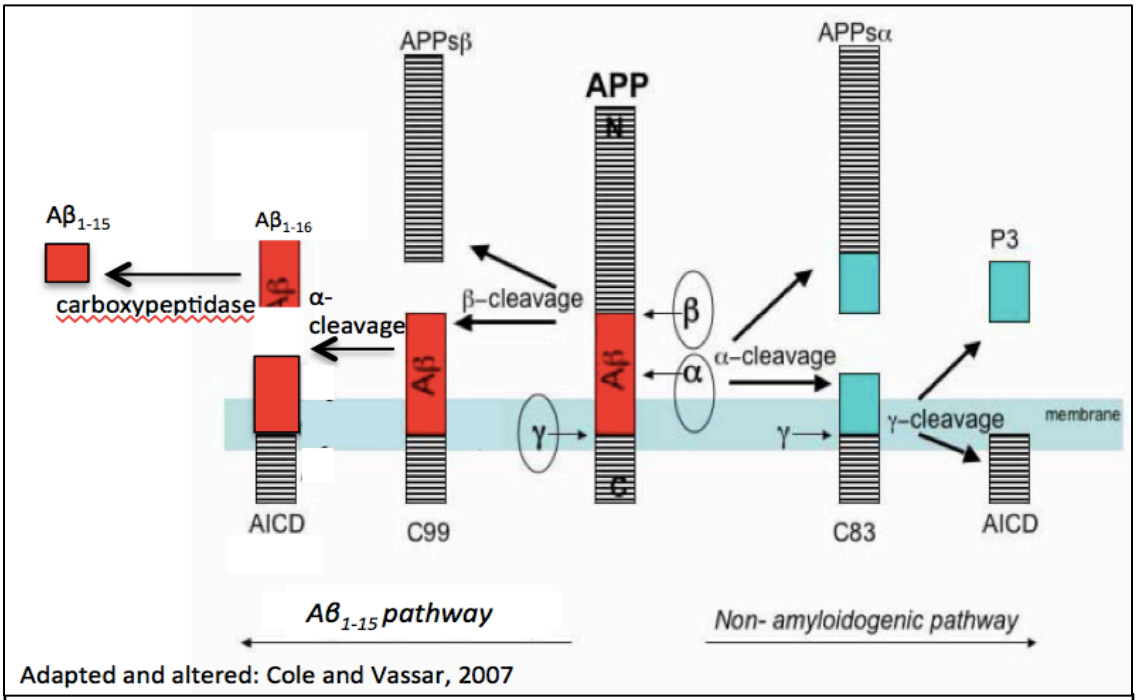

Figure 3. **$A\beta_{1-15}$ pathway**. $A\beta_{1-15}$ is produced by the cleavage of APP by β- and α-secretases followed by a carboxypeptidase.

followed by a carboxypeptidase (Fig.3).[10,28] This pathway was demonstrated by treating a cell culture model that secreted soluble APP and Aβ with γ-secretase inhibitors, which eliminated secretion of all Aβ isoforms larger than $Aβ_{1-16}$ (Fig. 4).[10,29] Three different studies in dogs, nonhuman primates, and humans confirmed the same result upon testing CSF after treatment with the γ-secretase inhibitor. The studies showed $Aβ_{1-15/16}$ increased up to 9 hours in dogs and humans and 48 hours in nonhuman primates after treatment.[30,31,32]

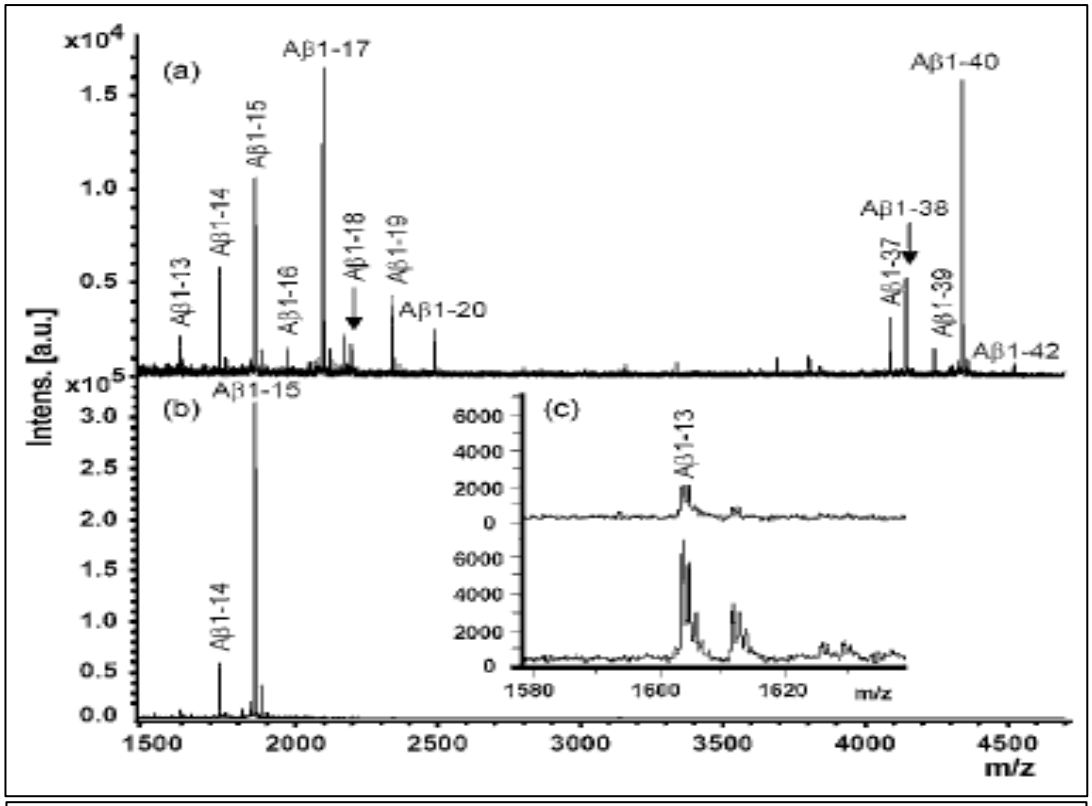

Figure 4. **A β isoform pattern without (a) or with (b) γ -secretase inhibitor.**[28] γ -secretase inhibitors eliminate secretion of all A β isoforms larger than A β_{1-16}

$Aβ_{1-15}$ is also increased in cerebrospinal fluid (CSF) of AD patients suggesting an alteration from the amyloidogenic pathway during pathogenesis.[10] The N-terminal fragment is hydrophilic and is extremely unlikely to contribute to aggregation of Aβ and

subsequent toxicity. Recent studies in our laboratory have demonstrated that $A\beta_{1-15}$ is a highly potent activator of α7 nAChRs, can enhance long term potentiation in hippocampal slices, can enhance memory of wild-type mice in contextual fear conditioning tests, and can rescue APPswe (an AD mouse model) LTP deficits.[2] Considering $A\beta_{1-15}$ is endogenous in CSF, is nontoxic, and highly active via nAChRs, $A\beta_{1-15}$ is a very attractive and promising peptide to test for neuroprotective effects against $A\beta_{1-42}$ toxicity.

Beta amyloid 10-15 ($A\beta_{10-15}$, Aβcore)

The 10-15 sequence of $A\beta_{1-15}$ (Fig. 5) was found to be accountable for the activity of both the N-terminal Aβ and $A\beta_{1-42}$ via calcium responses through its agonist-like action on nAChRs.[2] Whilst this shorter peptide is not produced endogenously, it has become a focal point of the current study because of the potential for development into a novel therapeutic for AD as a peptidomimetic. A short peptide and/or modified derivative would be much easier to develop as a drug that could potentially cross the blood-brain barrier (BBB) for non-invasive delivery to the area of interest, as it is currently unknown as to whether the $A\beta_{10-15}$ can cross the BBB. An alternative means for delivery would be application via the intranasal route.

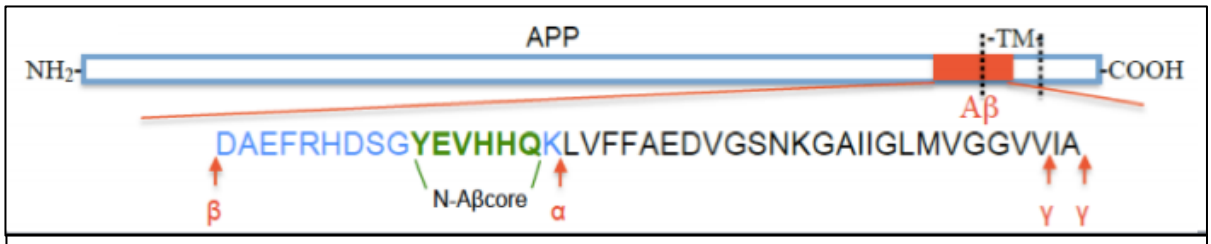

Figure 5. **Amino acid sequence of full length A β and fragments of interest.** A β_{1-42} whole sequence shown with A β_{1-15} in blue and green and A β_{10-15} in green.

AD and Aβ target receptors

Cholinergic dysfunction is a characteristic of AD pathology.[33,34] Acetylcholine and the cholinergic system play a pivotal role in synaptic plasticity and cognition. Changes and defects due to the dysfunction of the cholinergic system include changes in: acetylcholine synthesis, neurotropic factors, vesicular transporters, and muscarinic and nicotinic receptors.[33–35]

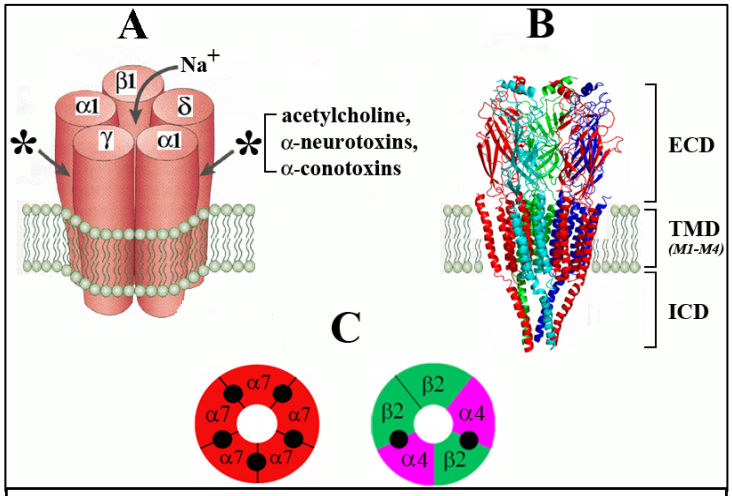

Figure 6. **Spatial organization of nAChRs**.[34] A $\beta_{1\text{-}42}$ has a very high binding affinity to nAChRs with agonist-like action through both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs. A, overview of the pentameric arrangement of the individual subunits of the nAChR in the membrane. B, side view of the atomic structure of the nAChR across the membrane (ECD: extracellular domain; TMD, transmembrane domain, ICD, intracellular domain. C, typical stoichiometric arrangement of individual subunits for the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs.

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated ion channels that are widespread in the central nervous system (Fig. 6).[36] They are activated by acetylcholine and nicotine and are made up of α- and/or β-subunits with the three most abundant subtypes in the brain being α7 receptors, α4β2 receptors, and α3β4 receptors.[35]

$A\beta_{1\text{-}42}$ has a very high binding affinity to nicotinic acetylcholine receptors (nAChRs), resulting an agonist-like action through both α7 and α4β2 nAChRs.[37,38,39]. Many studies have shown a link between nAChRs and AD, with the most vulnerable neurons being the ones that express these receptors.[35] Furthermore, our lab has demonstrated that

the presence of α4β2 nAChRs sensitizes NG108-15 model neuronal cells to the toxic effects of $A\beta_{1-42}$, further confirming this point.[39] Calcium imaging performed in our lab has also confirmed that both $A\beta_{1-42}$ and $A\beta_{1-15}$ are highly potent activators of α7 nAChRs with $A\beta_{1-15}$ having a significantly higher calcium response.[2] The nAChRs are a potential target for AD therapy because they play an important role in AD pathology with regard to Aβ binding.[33,35,40]

Aβ has several other putative target receptors, including cellular prion protein (PrPc), amylin receptors and metabotropic glutamate receptors, and p75 neurotrophin receptors, among others. Aβ binding to these receptors leads to changes in conformation and expression, and alterations in signaling pathways.[41,42] PrPc appears to be the primary receptor mediating toxicity with elevated Aβ levels. It is a glycoprotein that plays a pivotal role in synaptic structure and function. Aβ oligomers bind to cells expressing PrPc. PrPc has also been observed in Aβ plaques in brains of transgenic mice.[41–43]

NG108-15 cells

Our primary *in vitro* model is the NG108-15 hybrid rodent neuroblastoma cell line (mouse N18TG2 neuroblastoma cells fused with rat C6-BU-1 glioma cells). This cell line is able to form presynaptic-like varicosities after differentiation, which can form synapses with muscle cells, release acetylcholine, and have functional IP3- and ryanodine-sensitive calcium stores.[44–46] The NG108-15 cell line does not express nAChRs.

While Aβ accumulation in AD patients happens over many years, the simplified model systems used in this study allowed us to investigate the effects of Aβ through nAChRs over a shortened time frame. The ability to transfect the nAChRs into our model systems also allowed us to look at specific cells in culture to help distinguish responses caused by interaction of Aβ with nAChRs, as opposed to general Aβ toxicity, without transcriptional regulation. These model systems also allowed us to control the concentration of Aβ from low picomolar concentration, wherein Aβ acts as a neuromodulator, to nanomolar concentrations or higher causing dysfunction and eventual cell death. This simplified model allowed for direct investigation of $A\beta_{1-15}$ neuroprotection that could be quantified without the presence of secondary protective measures as found *in vivo*.

The ability to study $A\beta_{1-15}$ neuroprotection in a simple cell culture model provided a framework for advancing the study into hippocampal neuron cultures and, subsequently, into APPswe mouse models, with the long-term goal of evaluating $A\beta_{1-15}$ as a novel avenue for AD treatment.

Mouse Primary Hippocampal Neuron Cultures:

While *in vitro* cultures have been fundamental to the advancement of our understanding of the function of the nervous system, they have many limitations. Usually, the cell lines are derived from tumor cells to overcome the biggest limitation with primary neurons being post-mitotic and thus unable to divide (grow). Furthermore, these secondary cell lines, while useful as models, do not fully recapitulate the development and physiology

of the primary cells from which they are derived.[47,48] Primary cells solve this problem. Their primary advantage is that they replicate the properties of neuronal cells *in vivo*. Primary hippocampal neurons are able to extend axons and dendrites by a sequence typical of developmental events *in vivo* and are even able to form physiologically active synaptic contacts.[48,49] Their limitations are the practical issue of the typically small numbers of neurons obtained in cell culture preparations, even from several animals, and the restricted developmental stage from which viable cultures are usually prepared.

Alzheimer's Disease Pathology Mouse Models

Genetically modified mouse models are vital for uncovering the molecular mechanisms involved in AD pathology. Rodent models are appropriate due to functional modifications that mimic human pathology in the hippocampus and entorhinal cortex. Currently, a large number of different mouse models exist, varying as to the transgenes expressed for induction of AD pathology (primarily familial Alzheimer's disease (FAD) genes), specifically in regard to the timing and extent accumulation of Aβ.[50] Furthermore, behavioral phenotypes that present in accordance with insertion of FAD genes are relevant in view of the phenotypic similarity to human disease. Cognitive assays, performed in parallel to corresponding protein extractions from different cellular compartments to look at changes in protein levels for molecules of interest, can give us insight to the correlation of functional and molecular changes induced by different treatments.

Of note, there are only a limited number of other animal models for AD, most of which are either impractical or not readily available. Chief among these other models are canine and non-human primate dementia. The aged canine model for AD does have notable advantages, specifically similar late-onset pathology (ie. with aging alone), memory, cognitive and recognition deficits, and anxiety as that founds for humans [51]

As useful as the animal models are, there are limitations. The biggest limitation for the basic APP mouse models is that they are only able to produce amyloid plaques and, to a limited degrees, loss of neurons, but not neurofibrillary tangles that have a significant contribution to the pathogenesis of AD. It is also stressful for the animals to be surgically cannulated or injected. Another disadvantage is that breeding and housing of animals until the appropriate age proves to be costly. Despite these limitations, use of APP animal models is imperative for the uncovering of molecular changes that occur with Aβ accumulation in accordance with behavior in response to different treatments during different stages of pathophysiology of disease.

2. OBJECTIVE AND HYPOTHESIS

2.1. Objective

Beta amyloid (Aβ) is a key contributor to the pathogenesis of Alzheimer's disease (AD). It is produced via the sequential cleavage of the amyloid precursor protein (APP) by the β-site APP cleaving enzyme (BACE1 or β-secretase), followed by γ-secretase.[20] This processing sequence is known as the amyloidogenic pathway and results in the production of amyloid peptides 38-42 amino acids in length, owing to the variability in γ-secretase cleavage, with $A\beta_{1-42}$ being the dominant form present in neuritic plaques in the brains of individuals with AD.[14] $A\beta_{1-42}$ is produced in neurons and then released at synaptic sites. In normal brain, Aβ is present at relatively low levels (pM) in an oligomeric form and has been proposed to have a neuromodulatory role. During the onset of AD, excess Aβ accumulates to high nM to μM levels, most likely as the result of impaired clearance, and this pronounced increase correlates with disease progression. When left unchecked, Aβ appears to obstruct normal signaling and accumulate into amyloid plaques, eventually causing neuronal cell death.[52]

There is an alternative pathway by which APP is cleaved yielding N-terminal Aβ fragments 15-16 amino acids in length. Specifically, $A\beta_{1-15}$ is produced by the sequential cleavage of APP by β- and α-secretases, respectively, followed by carboxypeptidase.[10] Our lab has recently shown $A\beta_{1-15}$ to be highly active toward the nicotinic acetylcholine receptor (nAChR), as assessed by changes in neuronal calcium, and to modulate synaptic plasticity and contextual fear memory[3]. We have also shown that the presence of the high-affinity α4β2 nAChRs sensitizes neurons to the neurotoxic action of $A\beta_{1-42}$.[39]

In contrast, our most recent studies have shown the N-terminal Aβ fragment to be non-toxic. Due to its high activity and non-toxicity, we proposed therefore that $A\beta_{1-15}$ could have a neuroprotective action against $A\beta_{1-42}$-triggered neurotoxicity. The objective of this study was to address this possibility in various AD models: *in vitro*, *ex-vivo*, and *in vivo* to provide a potential new avenue for the development of novel AD therapies.

2.2 Central Hypothesis

The N-terminal Aβ fragment ($A\beta_{1-15}$) and the core hexapeptide fragment ($A\beta_{10-15}$) are neuroprotective against toxic full-length Aβ ($A\beta_{1-42}$). To test this hypothesis, the effect of treatment with $A\beta_{1-15}$ on $A\beta_{1-42}$–triggered neurotoxicity will be evaluated using *in vitro*, *ex-vivo* and *in vivo* approaches as follows.

3. SPECIFIC AIMS

3.1) SPECIFIC AIM 1. Characterize the neuroprotective effects of the N-terminal Aβ fragment against Aβ cytotoxicity.

***A. Elucidate the neuroprotective effects of the N-terminal Aβ fragment* in vitro .**

Specific Rationale: Sustained exposure of NG108-15 cells expressing nAChRs to nM levels of $A\beta_{1-42}$ causes increased reactive oxygen species and cell death. $A\beta_{1-15}$ is strongly activating through nAChRs with no toxic effects. We hypothesize that $A\beta_{1-15}$ is neuroprotective against $A\beta_{1-42}$ toxicity.

B. Examine the neuroprotective effects of the N-terminal Aβ fragment in ex-vivo primary hippocampal cell cultures.

Specific Rationale: It is important to compare *in vitro* results with *ex-vivo* hippocampal neuron cultures where the cells are more physiologically accurate and relevant to AD. *Ex-vivo* cultures recapitulate neuronal development and characteristics found *in vivo*.

3.2) SPECIFIC AIM 2. Study the neuroprotective action of $A\beta_{1-15}$ against $A\beta_{1-42}$-triggered deficits in synaptic plasticity.

Specific Rationale: Hormetic effects of $A\beta_{1-42}$ have clearly been shown wherein $A\beta_{1-42}$ enhances synaptic plasticity at low picomolar levels and blocks LTP at high nanomolar levels.[7] This has been supported via confocal calcium imaging in our lab, confirming the neuromodulatory role of $A\beta_{1-42}$.[2] Moreover, $A\beta_{1-15}$ shows even higher action via α7

nAChRs in calcium imaging experiments also supporting that this N-terminal fragment has neuromodulatory actions.[2] Taken together with the non-toxicity of this fragment, this led us to hypothesize that $A\beta_{1-15}$ will enhance LTP, and protect the synapses from $A\beta_{1-42}$ toxicity. We also hypothesize that $A\beta_{1-15}$ rescues LTP deficits in APPswe mice.

3.3) SPECIFIC AIM 3. Test the neuroprotective action of $A\beta_{1-15}$ against $A\beta_{1-42}$-triggered changes in fear memory and anxiety.

Specific Rationale: We have demonstrated that $A\beta_{1-15}$ has neuroprotective effects in several models of $A\beta_{1-42}$ toxicity (*in-vitro* NG108-15 cells, and *ex-vivo* primary hippocampal cell cultures and acute slices for LTP). We have also shown that $A\beta_{1-15}$ is able to enhance CFC in wild-type mice. Our goal in this Aim was to address the relevance of the neuroprotective action of $A\beta_{1-15}$ by demonstrating behavioral benefits in an intact Alzheimer's mouse model.

4. EXPERIMENTAL APPROACH AND MODEL SYSTEMS

Overall design: We exploited several model systems (NG108-15 cells, primary hippocampal cell cultures, and WT and AD mouse model) to investigate the neuroprotective effect of the $A\beta_{1-15}$ N-terminal fragment against $A\beta_{1-42}$ toxicity.

Relevance: The effect of neuroprotection by $A\beta_{1-15}$ from $A\beta_{1-42}$ toxicity provides a better understanding of how disruption by $A\beta_{1-15}$ can halt cell death and protect the cells. This may lead to new avenues for AD therapies.

Model systems: This study focused on three model systems: *in vitro* NG108-15 rodent hybrid neuroblastoma cells, *ex vivo* mouse hippocampal cell cultures, and *in vivo* WT, APPswe, and 5XFAD mouse models (further discussed in their respective sections). α4 & β2 nAChR plasmids were utilized to examine nAChR involvement in neuroprotection in NG108-15 cells. This allowed for a more direct understanding of $A\beta_{1-42}$ toxicity disrupted by $A\beta_{1-15}$ via nAChRs.

A β : Preparation of both $A\beta_{1-42}$, $A\beta_{1-15}$, and $A\beta_{10-15}$ was begun by reconstitution in double distilled water, bath sonication for 20 minutes, and then dilution to the working concentration in working solution as previously described.[2,39] All Aβ peptides were purchased from American Peptide and Peptide 2.0.

Approaches:

Reactive oxygen species (ROS): ROS assay was used to assess ROS production due to $A\beta_{1-42}$ and/or $A\beta_{1-15}$ action via the $\alpha4\beta2$ receptors. The Image iT live Reactive Oxygen Species (ROS) Detection kit (*Invitrogen*) was used to detect nonspecific ROS. 5-Carboxy-2',7'-dichlorodihydrofluorescein (carboxy-H_2DCFDA) enters the live cells and is deacetylated by non-specific esterases in the cell. Intracellular ROS oxidizes the reduced fluorescein, emitting green fluorescence visualized by fluorescence microscopy.

HOECHST staining: The blue fluorescent HOECHST 3342 dye (*Invitrogen*) is a nucleic acid stain that is highly cell-permeable and sensitive to chromatin conformation. HOECHST 3342 dye is visualized by fluorescence microscopy.

Electrophysiology: Basic hippocampal long-term potentiation (LTP) was performed as described in Lawrence et al., 2014.[2] Hippocampal slices were stimulated via the Schaffer collaterals and field potentials recorded from the CA1 pyramidal cells. Data are derived as slope values from the field potentials and are normalized to baseline.

Behavioral experiments: Contextual fear conditioning (CFC), Novel Object recognition (NOR) and Elevated Plus Maze (EPM) paradigms were employed to test mouse learning and memory. Single-trial CFC was performed as described by Sherrin et al., 2010 and Lawrence et al., 2014 in collaboration with Dr. Cedomir Todorovic and Dr. Tessi Sherrin.[2,53].

Western Blot: After behavior experiments, hippocampi were extracted, lysed and lysates collected and spun down to extract membrane vs. cytosolic proteins for Western (immunoblot) analysis.

Data and statistical analysis: Treatment and units were randomized as to order for all assays and experiments. Biological replicates were based on independent samples (*n*). Each experiment was repeated four times. Paired Student's *t*-test or one-way ANOVA with Bonferroni multiple comparison post-hoc test were performed for statistical analysis of different treatment groups for ROS, TUNEL, and Western blots associated with comparison of cell viability due to different treatment groups. Electrophysiology long-term potentiation experiments and behavior tests were also analyzed using one-way ANOVA with the Bonferroni posthoc test. All quantitative results are presented as boxplots (5-95% confidence intervals), where appropriate, or means ± SEM. All data were analyzed and graphed using Prism (Graphpad). *P*-values <0.05 were considered the minimum for significance (as rejection of the null hypothesis). Refer forward to appropriate chapters for detailed experimental design and data acquisition

5. SIGNIFICANCE

Approximately 5.2 million people in the United States are affected by Alzheimer's disease, and that number is expected to rise with increasing longevity.[5] Current FDA-approved drugs for AD treatment fall under two categories: Cholinesterase inhibitors (Donepezil and Galantamine), and NMDA receptor antagonists (Memantine).[54,55] These drugs generally treat the symptoms of AD by regulating neurotransmitters in the brain and there is an indication that Memantine may slow the progression of symptoms, but none is unable to change AD pathology. Therefore, it is important to examine other avenues for the development of AD therapies.

The amyloid hypothesis is based on the idea that the driving force in AD is the accumulation of $A\beta_{42}$ resulting from impaired clearance with age. Therapeutic targets based on this hypothesis could therefore be based on APP processing pathways. It would be possible to differentially target the APP processing pathways by inhibiting the amyloidogenic pathway via targeting of β- and γ-secretases or by enhancing the non-amyloidogenic pathway by targeting and promoting α-secretase activity. The problem with these strategies is that the secretases have a myriad of substrates and targeting them would cause too many negative symptoms.[54,56]

Portelius et al. (2011) demonstrated an alternative APP processing pathway that produces a variety of short Aβ peptides.[10] The $A\beta_{1\text{-}15}$ N-terminal fragment is of particular interest to this project because it is a short, hydrophilic peptide that does not aggregate[3]. It has been previously shown that Aβ has neuromodulatory effects at low levels and only becomes toxic at high levels.[8,9,55] Armed with this knowledge, our lab set

out to explore the possibility that $A\beta_{1-15}$ could also have neuromodulatory effects and found that it indeed was highly potent and more effective than full-length Aβ in agonist-like activity via nAChRs[3]. Pilot studies in a neuroblastoma cell line also showed that the $A\beta_{1-15}$ was nontoxic at the same concentrations that $A\beta_{42}$ showed clear toxicity leading us to the possibility that this N-terminal fragment could be neuroprotective. This study highlights the potential for development of a novel therapeutic for AD that would target the amyloid hypothesis by using endogenous peptides.

CHAPTER 2

CHARACTERIZATION OF THE NEUROPROTECTIVE EFFECTS OF THE N-TERMINAL Aβ FRAGMENT.

Introduction:

According to the amyloid hypothesis, the primary force driving AD is the prodromal accumulation of $A\beta_{42}$ in the brain. At picomolar levels, Aβ has a neuromodulatory role, but at high levels (micromolar) it becomes toxic leading to oxidative stress, cellular toxicity, synaptic dysfunction, and cognitive impairment.[39,21,9] Previously published work from our lab has shown that the presence of nicotinic acetylcholine receptors, specifically the α4β2 nAChRs, sensitize the cell to toxic effects of Aβ in such a way that disrupts calcium homeostasis, neuronal integrity, and mitochondrial function at picomolar to nanomolar levels.[39] In accordance with these studies, our lab has established an Aβ toxicity timeline representing key changes that occur in a neuroblastoma cell line transfected with α4β2 nAchRs (Fig. 7).[57] We are able to test

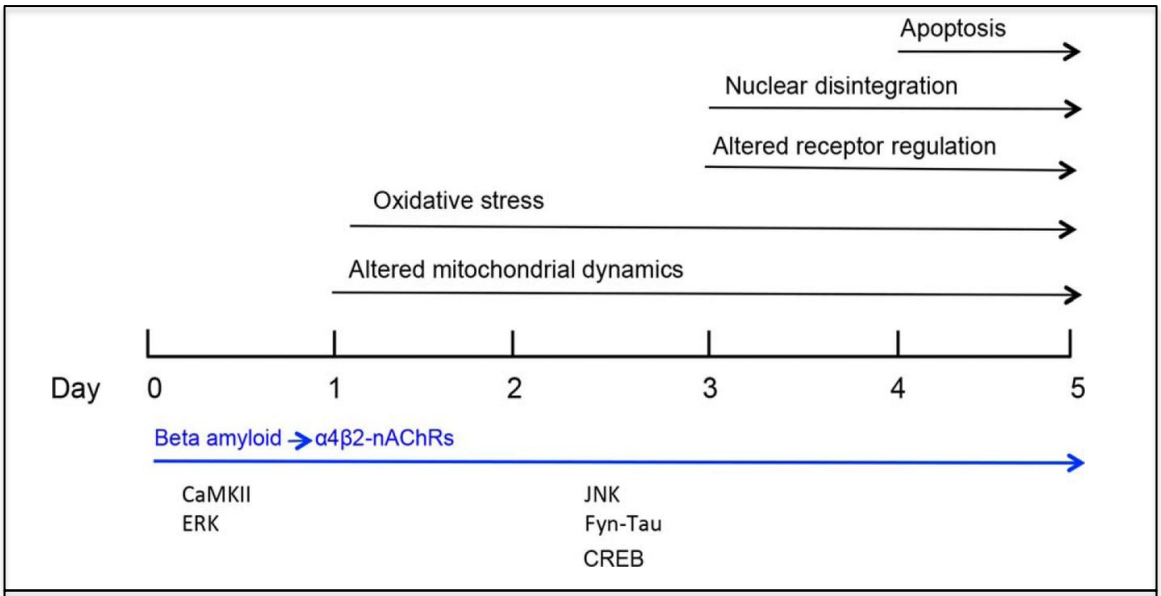

Figure 7. **A β toxicity timeline in α 4 β 2 nAchR- transfected NG108-15 cells**.[53] The effects of A β toxicity on NG108-15 cells are seen as early as day 1 with increases in oxidative stress and altered mitochondrial dynamics followed by nuclear disintegration and altered receptor regulation on day 3 and apoptosis beginning on day 4.

peptides and their ability to disrupt this toxicity at different time points corresponding to specific changes, or even to rescue from toxicity post treatment with $A\beta_{42}$.

In this study, we explored the neuroprotective potential of N-terminal Aβ peptides against the toxic effects of $A\beta_{42}$. We utilized an ROS assay to measure oxidative stress and examined nuclear disintegration as an early indicator of apoptosis to investigate the impact of different doses of $A\beta_{1\text{-}15}$ against $A\beta_{42}$ toxicity in the NG108-15 neuroblastoma model neuronal cell line. We also examined different treatment timing to explore how effective the N-terminal Aβ peptides are at reducing ROS and nuclear disintegration after $A\beta_{42}$ toxicity pathways are already activated. To determine whether the effects were long lasting, we also measured cell survival over one week of treatment.

We followed these experiments by tests in the more physiologically relevant primary hippocampal neuron culture system to confirm the neuroprotective effect of the N-terminal Aβ peptides. In the primary cultures, we can explore neuroprotection against Aβ toxicity via all receptors with which Aβ can interact.

$A\beta_{1\text{-}15}$ is postulated to be neuroprotective due to being still highly active via nAChRs, and as a short, hydrophilic peptide that is unlikely to aggregate.[2] The fact that it is an endogenous peptide also makes it an appealing molecule to explore and possibly develop as a novel therapeutic for AD.

A. Aim 1a. ELUCIDATE THE NEUROPROTECTIVE EFFECTS OF THE N-TERMINAL Aβ FRAGMENT *IN VITRO*

A.1. Experimental Design:

Model: Use of the differentiated NG108-15 hybrid neuroblastoma cell culture system in an Aβ neurotoxicity model has been well established.[39] We utilized this model to study the effects of $A\beta_{1-15}$ on cells exposed to toxic concentrations of $A\beta_{1-42}$ over three to five days using multiple methods, including ROS assay and HOECHST staining (see Fig. 7). The production of ROS is an indicator of oxidative stress, whereas the HOECHST staining permits assessment of the integrity of individual nuclei. The production of ROS is a general measure of cellular stress, while nuclear disintegration is a more direct measure of toxicity indicating the start of apoptosis. Cell survival was assessed with simple random cell counts over 7 days in both α4β2 nAChR-transfected and mock-transfected cells.

Transfection conditions: Before any experiments were performed, we had to identify the best-suited transfection reagent that caused the least toxicity in culture with the maximum transfection rate ("efficiency"). We did this by performing immunocytochemistry using α4 nAChR subunit primary antibody to look at both α4*-nAChR immuno-positive cells (% immune positive) and signal intensity measured via ImageJ. We also utilized a HOECHST stain to look at toxicity of the reagents via nuclear disintegration. For transfection reagents, we tested both FuGENE (Promega), and NeuroMag (OZ Biosciences) with 2uL DNA:5ul Neuromag or 2uL DNA:6uL Neuromag with the following results:

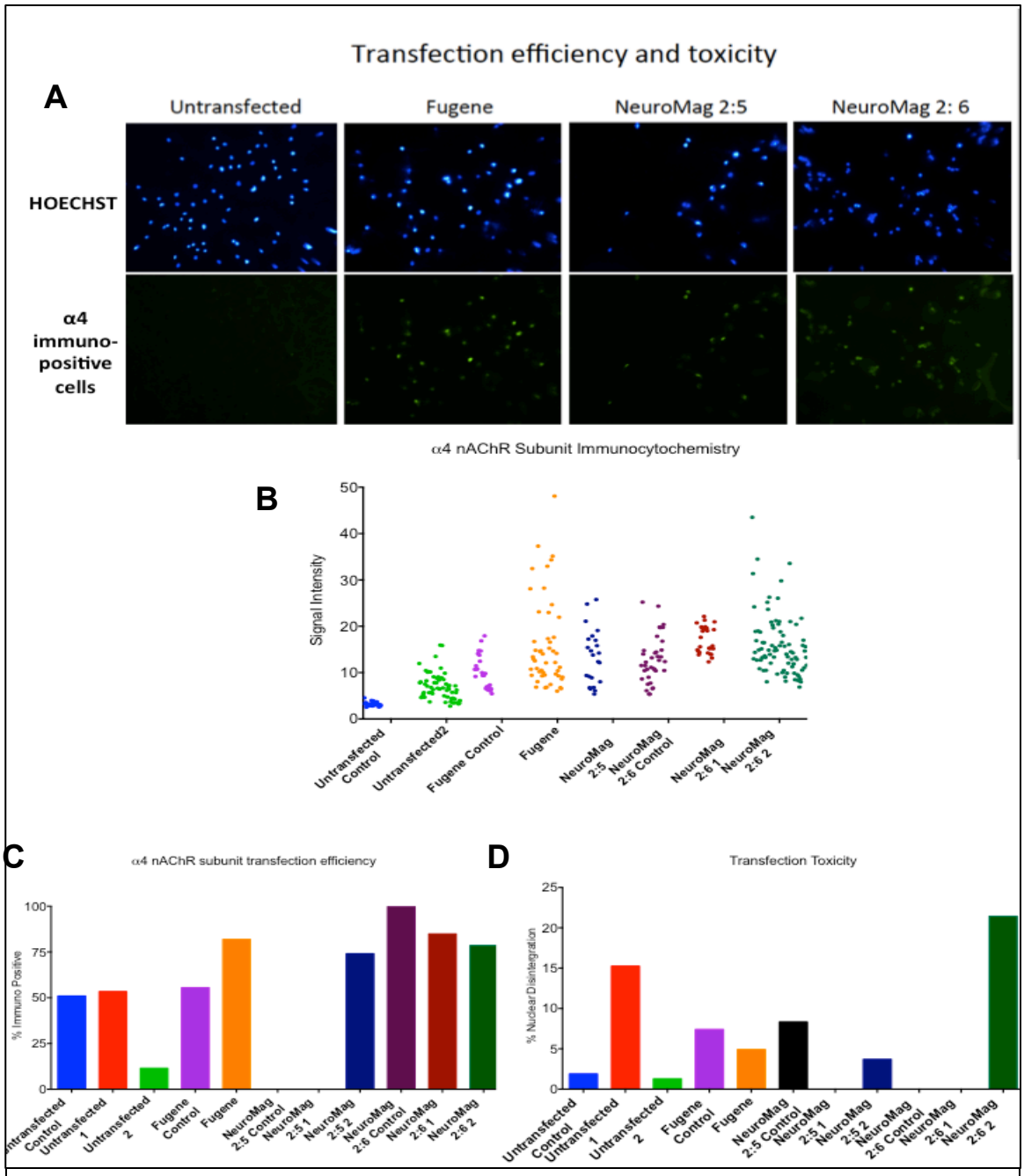

Figure 8. **Fugene has the highest transfection efficiency and lowest toxicity in NG108-15 cells.** Transfection efficiency and toxicity confirmed via α 4-immunocytochemistry and Hoechst staining. Transfection conditions tested were: FuGENE and NeuroMag with 2uL DNA:5ul Neuromag or 2uL DNA:6uL Neuromag. A, Respresentative micrographs of each transfection condition. B, Signal intensity measured via imageJ. C, Percent immune-positive cells calculated via raw cell counts of immunopositive cells compared to total cells. D, Percent nuclear disintegration calculated by counting fragmented nuclei utilizing Hoechst stain.

Although NeuroMag 2:6 produced signal intensity and α4-nAChR immunopositivity that matched that found for FuGENE, the Neuromag 2:6-transfected cultures displayed higher levels of baseline nuclear disintegration. This confirmed that FuGENE would be the most appropriate for transfecting the α4 and β2 nAChR subunit pcDNA vectors.

Timing and Dosage: We performed 2 sets of three different experiments looking at $A\beta_{1-15}$ treatment timing as well as different $A\beta_{1-15}$ dosage, to investigate the effect of $A\beta_{1-15}$ on $A\beta_{1-42}$ toxicity. All experiments had the following treatments: Untreated, 100nM $A\beta_{1-42}$, $A\beta_{1-15}$, combination 100nM $A\beta_{1-42}$ and (different concentrations) $A\beta_{1-15}$, 50μM N-acetylcysteine (NAC), and combination 100nM $A\beta_{1-42}$ with 50μM NAC. NAC is used as a generic antioxidant. It is a precursor to the formation of glutathione, an antioxidant that can reduce free radicals.

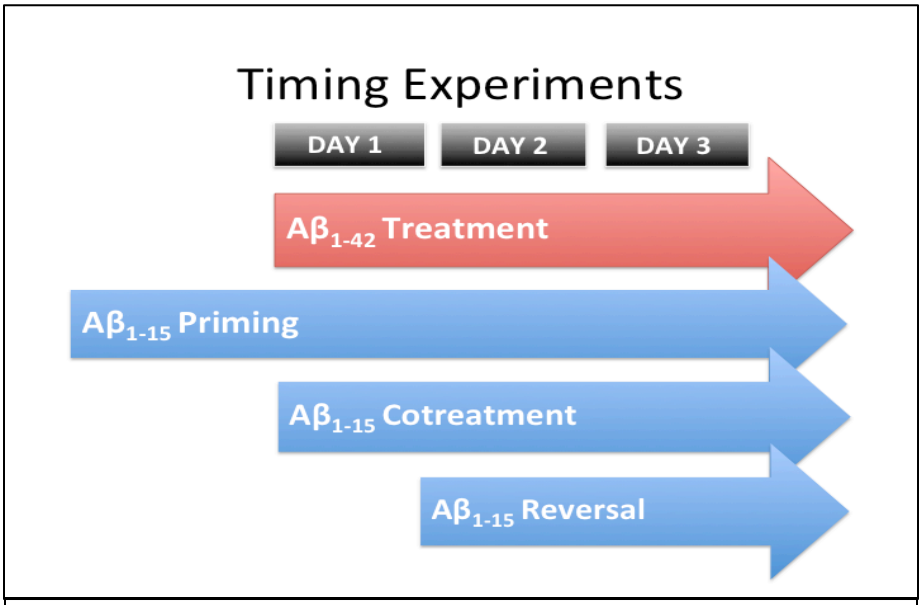

Figure 9. **Timing Experiment Schematic.** Different timing of 100nM $A\beta_{1-15}$ (Priming- 1 day prior to $A\beta_{1-42}$, Cotreatment and Reversal- 1 day post $A\beta_{1-42}$ treatment) will be tested in combination with 100nM $A\beta_{1-42}$ to investigate timing effect of $A\beta_{1-15}$ neuroprotection against $A\beta_{1-42}$ toxicity.

1. The combination treatment timings were varied for each experiment (Fig. 9).

- To explore potential competition at the level of the target receptors, the combination treatments were done at the same time.
- For priming experiments to examine the impact of pretreatment with the fragment, $A\beta_{1-15}$ or NAC was added one day before $A\beta_{1-42}$,
- For reversal/rescue experiments the $A\beta_{1-15}$ and NAC combination with $A\beta_{1-42}$ was added one day after $A\beta_{1-42}$.

2. Dosage experiments were carried out to examine the neuroprotective potency of the N-terminal fragment $A\beta_{1-15}$ on $A\beta_{1-42}$ toxicity. We explored 3 different concentrations: 10nM, 100nM, and 500nM $A\beta_{1-15}$ on 100nM $A\beta_{1-42}$ (Fig. 10), based on previous experiments examining the potency of the Aβ peptides in neuromodulation.

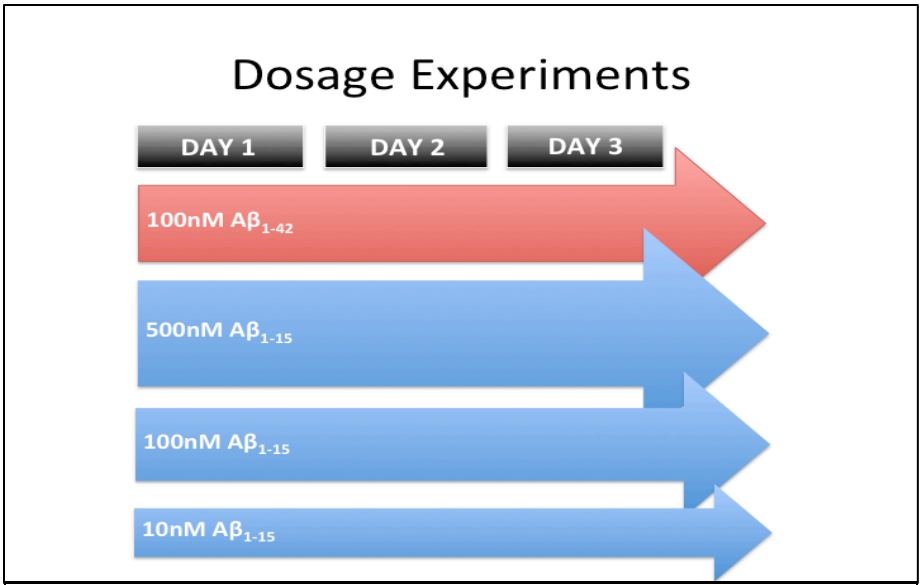

Figure 10. **Dosage Experiment Schematic.** Different dosages of A β $_{1-15}$ (10, 100 and 500nM) will be tested in combination with 100nM A β $_{1-42}$ to investigate potency of $A\beta_{1-15}$ neuroprotection against $A\beta_{1-42}$ toxicity.

ROS assay and HOESCHT staining were performed on the fourth day to assess the effects of $A\beta_{1-15}$ on $A\beta_{1-42}$ toxicity and were compared to untreated controls and NAC treatment. This approach directly tested the neuroprotective properties of the N-terminal Aβ fragment. The ROS assay showed general ROS, which parallels the oxidative stress the cells are incurring due to $A\beta_{1-42}$ toxicity. The Hoechst stain, on the other hand, identifies cells that are in the early stages of apoptosis, because it directly stains DNA, with fragmented nuclei indicating progression toward cell death. This allowed us to investigate different stages of the compromise of cell integrity due to the toxicity of $A\beta_{1-42}$. Stains were visualized on the Olympus IX71 epifluorescence microscope via a Macrofire camera.

In addition, a cell survival experiment was performed to examine the length of time the cells will survive the different treatments and to observe whether the neuroprotective effect of $A\beta_{1-15}$ is sustained for over a week.

A.2. Materials and Methods:

Cell Culture:

NG108-15 cells were first plated on poly-L-lysine-coated or Cell-Tak-coated coverslips in 35mm dishes in 15% fetal bovine serum (FBS) with Dulbecco's modified Eagle's medium (DMEM). After allowing for the cells to attach (approximately 2 hours), the media was changed to DMEM with 1% FBS and 1mM dibutyryl cyclic AMP (differentiation media) to induce neuronal differentiation.

Forty-eight hours after differentiation, when the cells had developed neurites and varicosities, mouse α4 and β2 nAChR sequences in pcDNA 3.1 vectors were transfected into the cells at a 1:4 ratio using FuGENE reagent. Mock transfection was carried out using FuGENE reagent with no plasmid. Expression of nAChRs was verified by α4-nAChR immunocytochemistry up to one week post-transfection.

Treatments:

Treatments began 72 hours after transfection and consisted of the aforementioned treatment groups (Figs. 9&10). Cell culture media was changed every day with the appropriate treatments to keep concentrations of treatments consistent throughout the treatment days. Treatments lasted for 3 days with ROS assay and Hoechst staining performed on the 4^{th} day.

ROS and HOECHST staining:

The Image iT live Reactive Oxygen Species Detection kit (Life Technologies) was utilized with Hoechst staining as described under the experimental approach. The assay was performed in accordance with the manufacturer's protocol. After the last day of treatment the cells were washed with HBSS and then incubated with Component A (carboxy-H_2DCFDA) at 37°C for 30 minutes. Component B (HOECHST stain) was added in the last 5 minutes of incubation at 2µg/mL. Finally, cells were washed with HBSS and visualized via Olympus IX71 epifluorescence microscope linked to a Macrofire camera at 495 nm excitation/529 nm emission for ROS and 350 nm excitation/461 nm emission for HOECHST stain.

Data were graphed as percent ROS positive cells by doing simple cell counts from randomly chosen fields of view with a minimum sample size of four for each test. Nuclear disintegration was similarly quantified.

Cell Survival:

We also performed a 7-day cell survival assay under previously outlined treatments, to better elucidate the toxicity/neuroprotective effects of different treatments, with media changed out and bright field pictures taken daily utilizing the Olympus IX71 microscope system. These were simple cell counts taken from 3 random fields of view per treatment (3 replicates) for 7 days. Data were graphed as percent cell survival ((number of remaining cells/number of cells on start day)*100). The minimum sample size was four for each treatment group.

A.3. Results

We established a toxicity timeline in differentiated NG108-15 cells transfected with nAChRs and treated with full length Aβ (Fig. 7; ref 40). To examine the neuroprotective potential of $A\beta_{1-15}$, we measured ROS and disintegrated nuclei in cultures co-treated with both $A\beta_{1-42}$ and $A\beta_{1-15}$ in different dosage and timing combinations.

For priming experiments, we showed that the $A\beta_{1-15}$ pretreatment significantly blocked the action of $A\beta_{1-42}$ via nAChRs as evidenced by the low ROS staining and reduced nuclear disintegration, either by competing for Aβ and/or activating cell survival pathways (Fig. 11, $p<0.001$). For the co-treatment experiments, the $A\beta_{1-15}$ blocked $A\beta_{1-42}$ and any associated downstream effects also resulting in low ROS and nuclear disintegration (Fig. 11, $p<0.001$). Surprisingly, neuroprotection in the delayed treatment (rescue) group was also significant (Fig. 11, $p<0.01$), though to a lesser degree than the co-treatment and priming groups. This rescue occurred despite the initiation of the cellular toxicity process by full-length $A\beta_{1-42}$, namely increased ROS and nuclear disintegration indicating the start of apoptosis.

The dose-response experiments produced unexpected results. Based on our electrophysiology studies that show very high potency of $A\beta_{1-15}$ on synaptic plasticity (ref), we anticipated that $A\beta_{1-15}$ would still have neuroprotective effects at very low nM levels. At 10nM, $A\beta_{1-15}$ was not able to significantly reduce ROS production with the 100nM $A\beta_{1-42}$ co-treatment (Fig. 11). However, both the 100nM and 500nM $A\beta_{1-15}$ co-treatment were able to significantly prevent ROS production (Fig. 11). Furthermore, our

antioxidant control, NAC reduced ROS similarly to $A\beta_{1-15}$ and untreated control levels, confirming the staining was the result of ROS and not reactive nitrogen species.

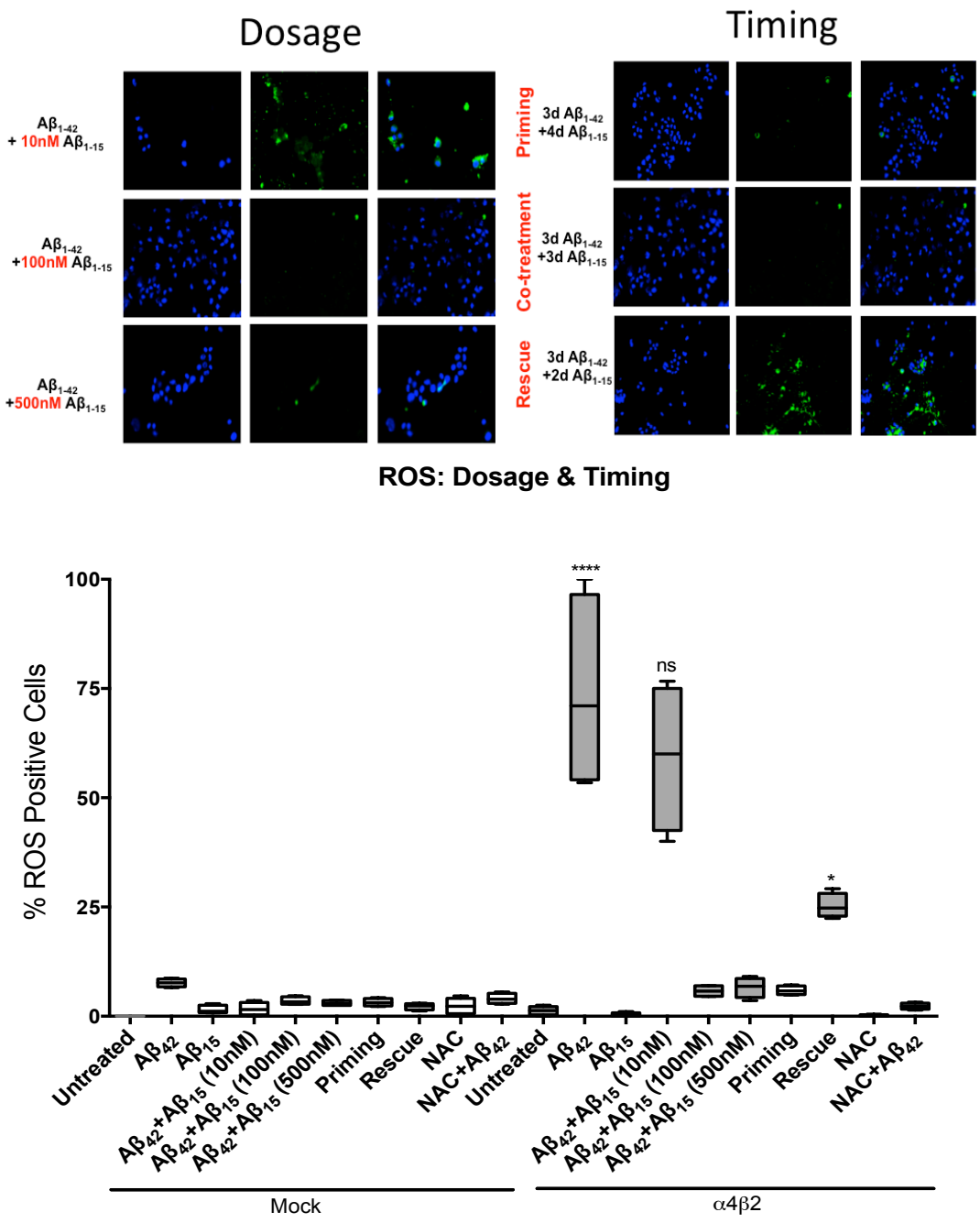

Figure 11. **The N-terminal Aβ_{1-15} protects against Aβ-induced oxidative stress.** Co-treatment with Aβ_{1-15} at 10, 100 and 500nM. Priming: Treatment with Aβ_{1-15} 1d prior to the start of co-treatment with Aβ_{1-42}. Rescue: Co-treatment with Aβ_{1-15} 1d subsequent to the start of treatment with Aβ_{1-42}. 100 and 500nM as well as priming, rescue and co-treatment protect against Aβ toxicity. *p<0.05 ****p<0.0001 as compared to mock-transfected cells in one-way ANOVA with post-hoc test. Data are presented as 5-95 percentile box-and-whisker plots, n=4 experiments/group from 3 random fields of view with 3 replicates per experiment.

Nuclear disintegration data showed that combination treatments with $A\beta_{1-15}$ or NAC are able to prevent nuclear disintegration caused by $A\beta_{1-42}$ toxicity for all of the dosage and timing treatments (Fig. 12, $p<0.0001$). Unlike the oxidative stress data, even the 10nM $A\beta_{1-15}$ co-treatment with 100nM $A\beta_{1-42}$ was enough to prevent nuclear disintegration caused by $A\beta_{1-42.}$ (Fig. 12), indicating that it may be sufficient to block apoptosis.

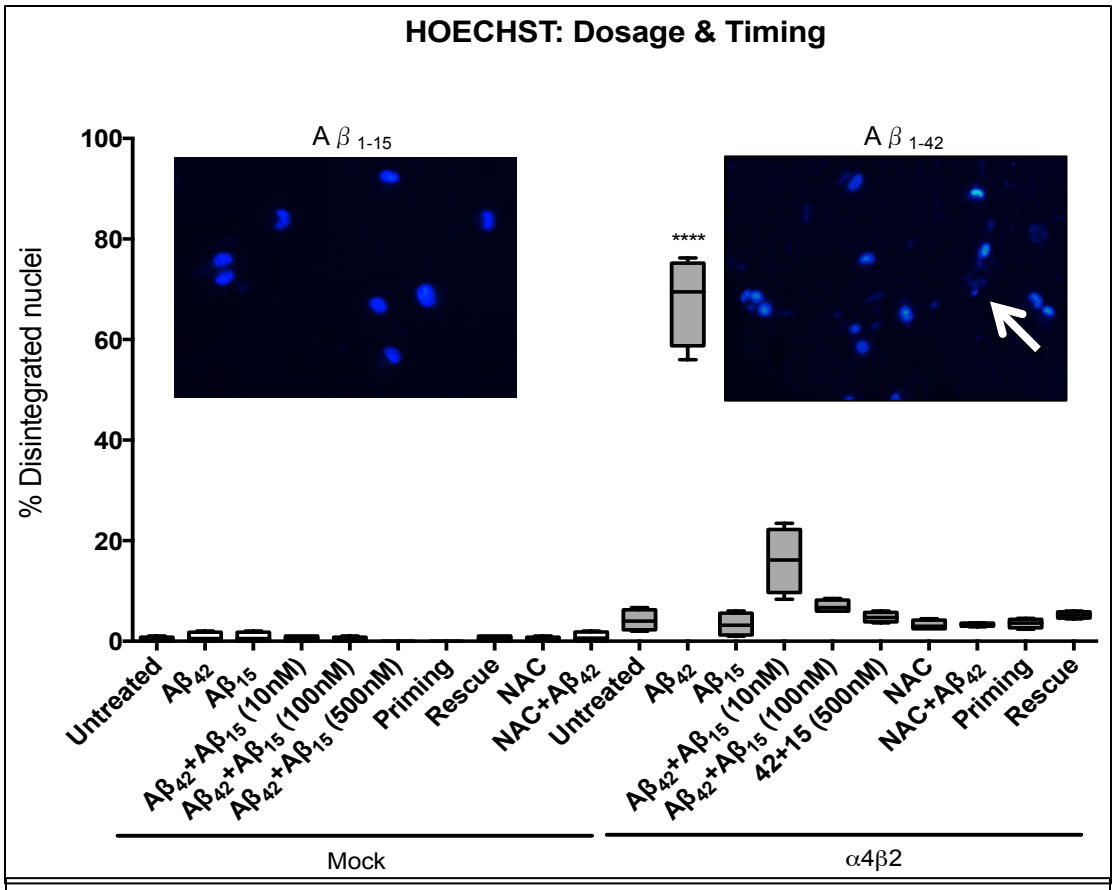

Figure 12. **The N-terminal A β $_{1-15}$ protects against A β -induced nuclear disintegration:** Conditions as described for Fig. 10. All co-treatment dosage and timing conditions protected against A β -induced nuclear disintegration. Insets showing representative micrographs of cultures treated with A β $_{1-15}$ or A β $_{1-42}$ with arrow pointing to disintegrated nuclei. ****$p<0.0001$, .one-way ANOVA with post-hoc test. Data are presented as 5-95 percentile box-and-whisker plots, n=4/group.

Cell survival experiments demonstrate more accurately the timeline of cell death in differentiated NG108-15 cultures. Cells transfected with nAChRs and treated with $A\beta_{1-42}$ die faster than non-transfected $A\beta_{1-42}$-treated cells. Further co-treatment with either $A\beta_{1-15}$ or $A\beta_{core}$ is neuroprotective against this toxicity (Fig. 13, $p<0.0001$ compared to $A\beta_{1-42}$). These changes were first detectable on day 5 when there was a significant decrease in cell survival in α4β2 nAChR-transfected cultures treated with $A\beta_{1-42}$ (Fig. 13, $p<0.05$), followed by decreased survival of the mock-transfected cultures treated with $A\beta_{1-42}$ on day 6 (Fig. 13, $p<0.001$). This trend continued on to 7 days with both the transfected and mock-transfected NG108-15 cells treated with $A\beta_{1-42}$, showing significantly higher cell death compared to combination treated cells with $A\beta_{1-15}$ or $A\beta_{CORE}$ alone, or in combination with $A\beta_{1-42}$ (Fig. 13, $p<0.0001$).

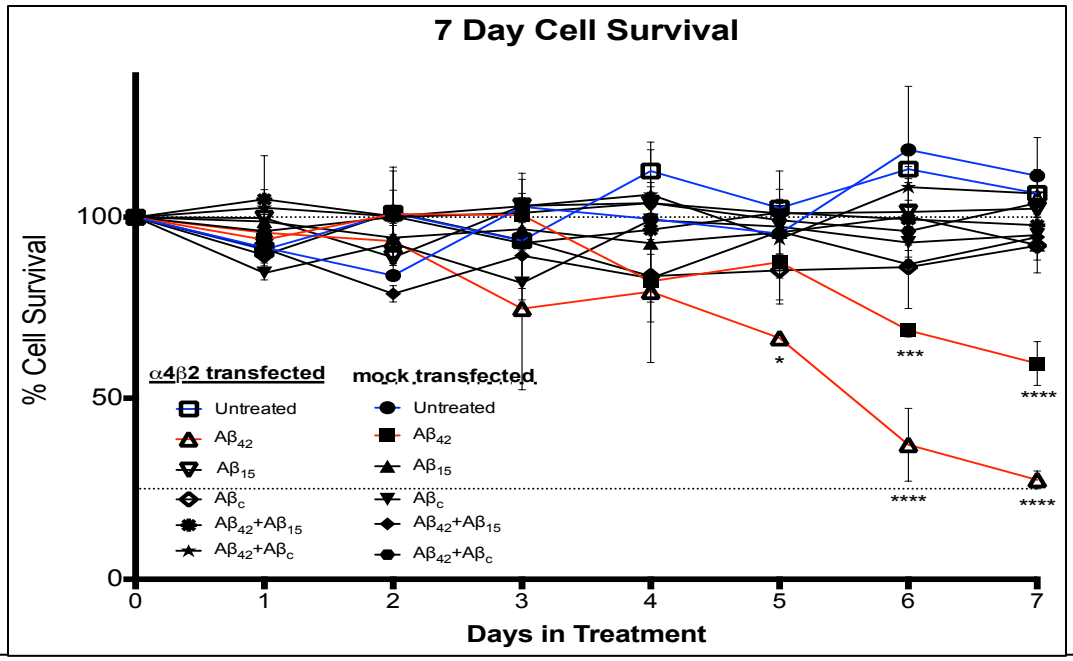

Figure 13. **The N-A β fragment and the N-A β core protect against A β_{1-42} -induced cell death in NG108-15 cell culture.** A, Cell counts in NG108-15 cells with, or without, $\alpha4\beta2$-nAChRs treated daily with 100nM A β_{1-42}, N-A β fragment or N-A β core alone, or combination treatments with A β_{1-42} and N-A β fragment or N-A β core over 7 days (n=4). (*p<0.05) (***p<0.001) (****p<0.0001). Data are means ± SEM, n=4 experiments/group from 3 random fields of view with 3 replicates per experiment.

B. Aim 1b EXAMINE THE NEUROPROTECTIVE EFFECTS OF THE N-TERMINAL Aβ FRAGMENT IN *EX-VIVO* PRIMARY HIPPOCAMPAL CELL CULTURES.

B.1. Experimental Design

For primary hippocampal cell cultures, there are several steps we needed to resolve to prepare the cells for neuroprotection experiments. The experimental plan was as follows:

1. First, it was necessary to establish neonatal mouse hippocampal cell cultures in which $A\beta_{1-42}$ toxicity could be identified. Approximately 6 days in culture under selection for neurons using Neurobasal media with B57 supplementation was sufficient for the majority of cells to assume polygonal somas and extend long

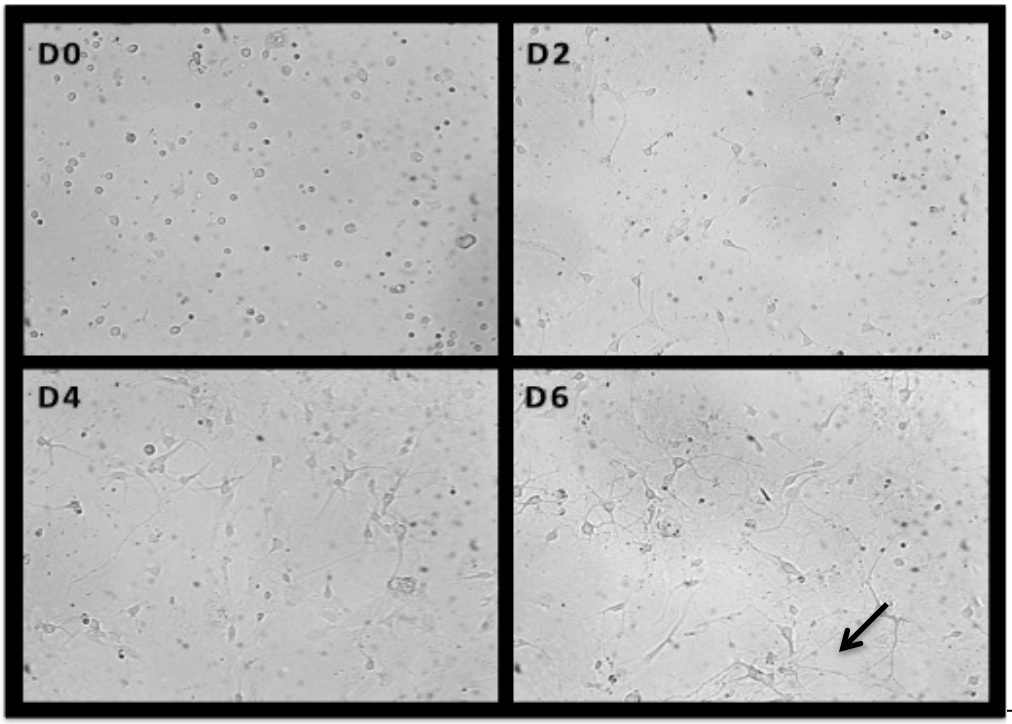

Figure 14. **Primary hippocampal culture development over 6 days.** 20x micrographs showing development of primary hippocampal neuron cultures taken from 1d old WT mouse over 6 days in culture. Arrow pointing to pyramidal neuron in culture.

neurites typical of cultured neurons. Many of the neurons showed pyramidal neuron-like morphologies very similar to pyramidal cells *in vivo*. This is consistent with the continuation of hippocampal neuron development *in vitro* in parallel to *in vivo* development (Fig. 13).[48]

2. Next, we established a timeline for $A\beta_{1-42}$ toxicity in the neuronal cultures. This was achieved by treating the cultures with a high concentration of $A\beta_{1-42}$ over several days to weeks. ROS and cell counts were used to assess cell culture oxidative stress and viability at different treatment days to determine when the cells start deteriorating, and finally, cause most of the primary culture to diminish. Once the timeline was set, we were able to investigate the neuroprotective effects of $A\beta_{1-15}$.
3. Finally, we explored neuroprotective effects of $A\beta_{1-15}$ on hippocampal cell cultures. This was accomplished by treating the cell cultures with different concentrations of $A\beta_{1-15}$ in combination with full length Aβ.

B.2. Materials and Methods:

Animals:

Neonatal B6.SJL mice (Jackson Labs) between 0-2 days old were used for primary hippocampal cell culture. All animals used were in accordance with approved IACUC protocols and animal welfare guidelines.

Primary Hippocampal Culture:

Hippocampal cell cultures were prepared from 0-2 day old mice as described by Cheng, et al.[58] Neonatal mice of either gender were swiftly decapitated, and the brains quickly extracted from the skull and placed in ice-cold Neurobasal A medium with 5% fetal bovine serum, Gentamicin and B27 supplement (serum NB). Hippocampi were then isolated using a dissecting microscope, minced and digested in a papain solution (Worthington, LS003126, Lot # 35N16202) for 15 minutes in a 37°C incubator. The cells were then centrifuged and washed twice with serum NB, and then serially triturated with three polished Pasteur pipettes of decreasing diameter to dissociate the cells. The primary cells were then centrifuged one last time, washed with serum NB and plated on 24-well plates coated with poly-D-lysine (after 15 minute pre-plating to remove adherent glial cells). Cells were maintained in Neurobasal A medium with B27 and Gentamicin and treatment begun after 1 week in culture, spanning 5 or 10 days with ROS and cell survival experiments as described in Aim 1a.

Treatments:

The Aβ toxicity timeline was established by treating primary cultures with micromolar Aβ concentrations over several days. ROS Image iT Live assay kit was utilized and visualized as described in Aim 1.

B.3. Results

We were able to successfully culture primary hippocampal cells and establish an $A\beta_{1-42}$ toxicity timeline in these cultures. Early experiments indicated the need to treat the cell cultures with higher (μM) doses of $A\beta_{1-42}$, compared to the lower nM doses used in differentiated NG108-15 cells expressing nAChRs, in order to induce measurable toxicity.

After five days of treatment there was already a significant difference in ROS between $A\beta_{1-42}$ treatments compared to all other treatments (Fig. 15, $p<0.0001$). Both the $A\beta_{1-15}$ and $A\beta_{core}$ combination treatments with $A\beta_{1-42}$ were able to protect the primary

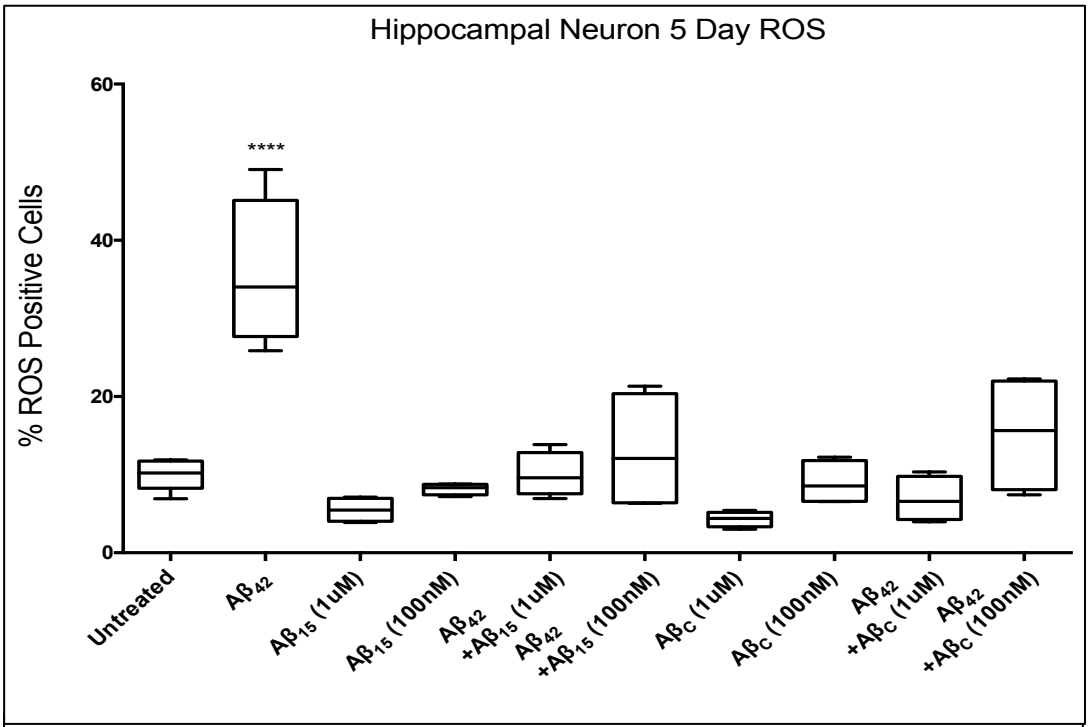

Figure 15. **The N-A β core and the N-A β fragment protect against A β $_{1-42}$-induced oxidative stress in primary hippocampal neurons**. 5-day treatments with 1μM A β $_{1-42,}$ 1μM and 100nM N-A β fragment, or 1μM and 100nM N-A β core and combination treatments of 1μM A β $_{1-42}$ and 1μM or 100nM N-A β fragment or 1μM or 100nM N-A β core (*n*=4). Percent positive ROS cells represented by 5-95 percentile box-and-whiskers plots (n=4 experiments/group from 3 random fields of view with 2 replicates per experiment.

cells from this toxicity, with ROS levels comparable to untreated control levels (Fig. 15, $p<0.0001$, compared to $A\beta_{1-42}$). Interestingly, in the primary hippocampal cells, the 100nM $A\beta_{1-15}$ or Aβcore treatments in combination with 1uM of $A\beta_{1-42}$ were still able to significantly prevent ROS production due to $A\beta_{1-42}$ toxicity (Fig. 15, $p<0.0001$)

Ten-day treatments produced results similar to the 5-day treatments with differences becoming even more pronounced between the different treatment groups. $A\beta_{1-42}$-treated groups exhibited higher levels of ROS (compared to 5-day treatment) and with combination-treated groups produced lower levels, in some cases comparable to untreated control levels (Fig. 16, $p<0.0001$ untreated and combination-treated groups compared to $A\beta_{1-42}$).

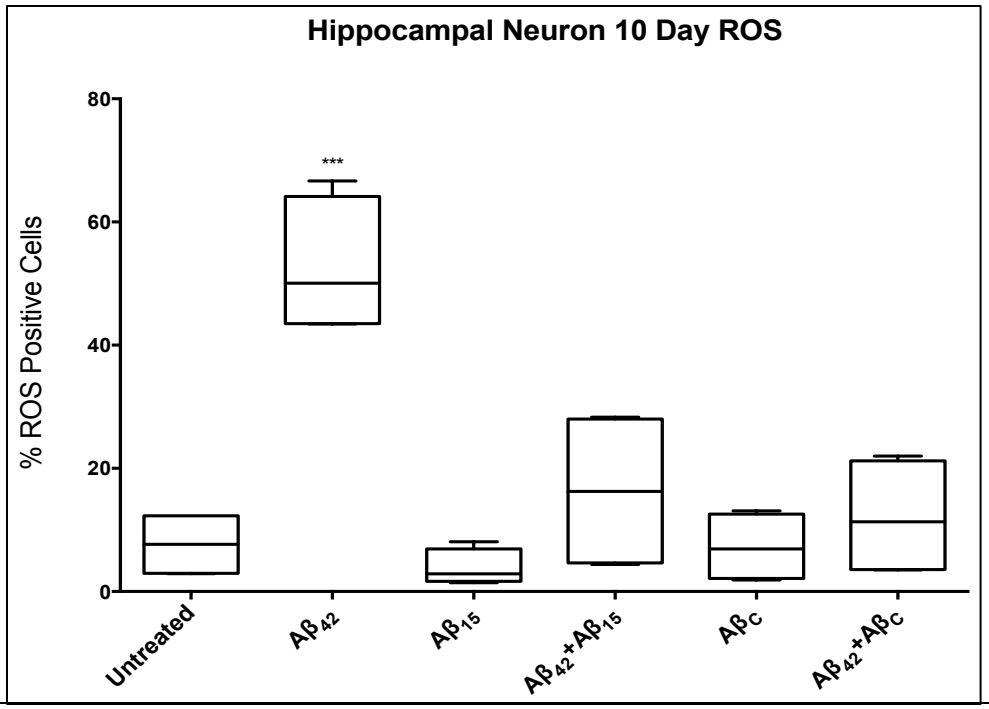

Figure 16. **The N-A β core and the N-A β fragment protect against A β_{1-42}-induced oxidative stress in primary hippocampal neurons**. 10-day daily treatments with 1μM A $\beta_{1-42,}$ 1μM N-A β fragment, or 1μM N-A β core and combination treatments of 1μM A β_{1-42} and 1μM N-A β fragment or 1μM N-A β core (*n*=4). Percent positive ROS cells represented by 5-95 percentile box-and-whiskers plots (n=4 experiments/group from 3 random fields of view with 2 replicates per experiment. ***p<0.0001 (Tukey *post hoc* tests)

We also explored glutamate toxicity to examine whether $A\beta_{1-15}$ or $A\beta_{10-15}$ are able to protect against excitotoxicity and saw similar levels of ROS production as $A\beta_{1-42}$ at five days of glutamate treatment at 100μM. Glutamate (100μM**)** treated cultures caused significantly high oxidative stress compared to untreated controls (Fig. 17, $p<0.0001$) with significant reduction by combination treatment with $A\beta_{1-15}$ at 1μM (Fig 17, $p<0.001$). All other combination treatments showed no significant reduction of ROS.

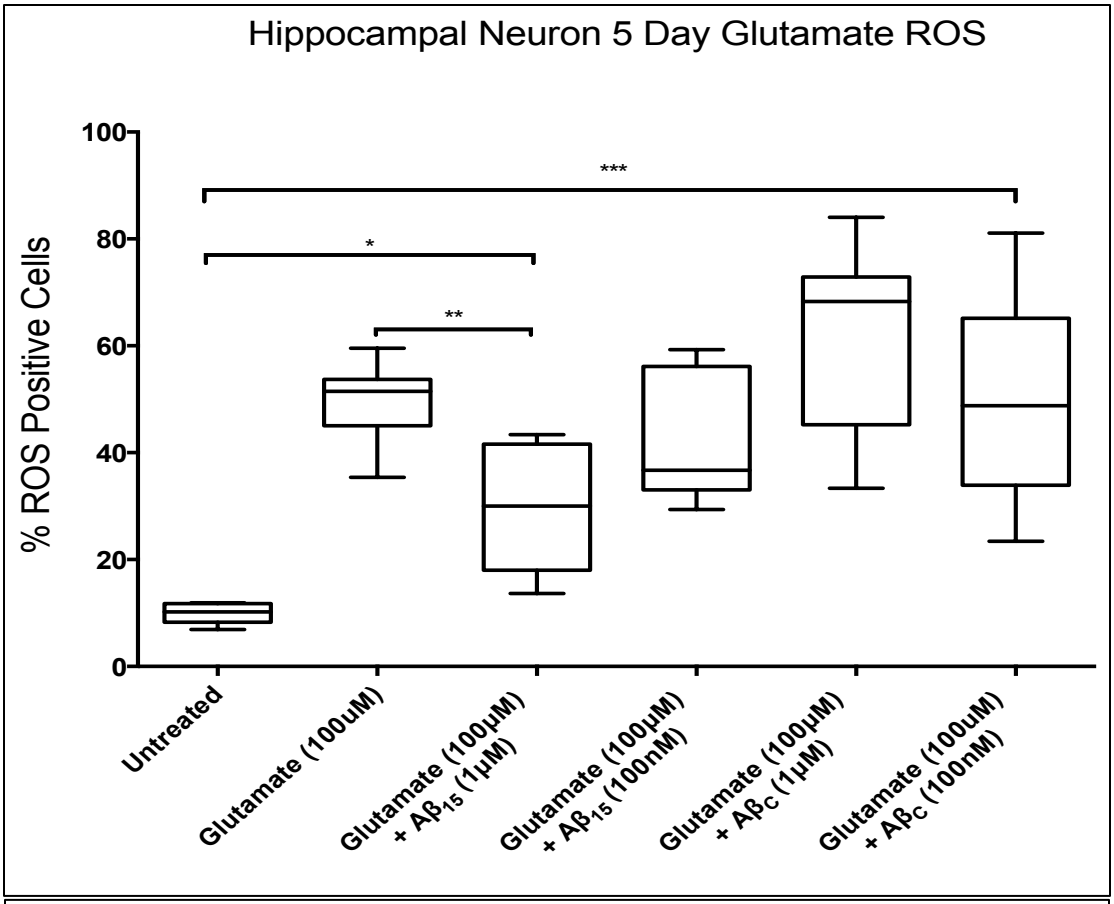

Figure 17. **The N-Aβcore and the N-Aβ fragment are unable to protect against glutamate-induced oxidative stress in primary hippocampal neurons**. 5-day treatments with 100μM Glutamate, 1μM and 100nM N-Aβ fragment, or 1μM and 100nM N-Aβcore and combination treatments of 100μM Glutamate and 1μM or 100nM N-Aβ fragment or 1μM or 100nM N-Aβcore (*n*=4). Percent positive ROS cells represented by 5-95 percentile box-and-whiskers plots (n=4 experiments/group from 3 random fields of view with 2 replicates per experiment. ***p<0.0001 (Tukey *post hoc* tests)

Cell survival experiments taken out to 10 days give us a better understanding of the neuroprotective effect of these treatments on the primary neurons. There was a much faster rate of cell death in $A\beta_{1-42}$ treated cells compared to the other groups. Both $A\beta_{1-15}$ and $A\beta_{core}$ were protective against this $A\beta_{1-42}$ toxicity. At day 5 we detected a significant decline in cell numbers when treated with $A\beta_{1-42}$ (Fig. 18, $p<0.0001$), which continued to decline until day 10. This cytotoxicity was prevented by co-treatment with either $A\beta_{1-15}$ or the core fragment $A\beta_{10-15}$ (Fig. 18, $p<0.0001$).

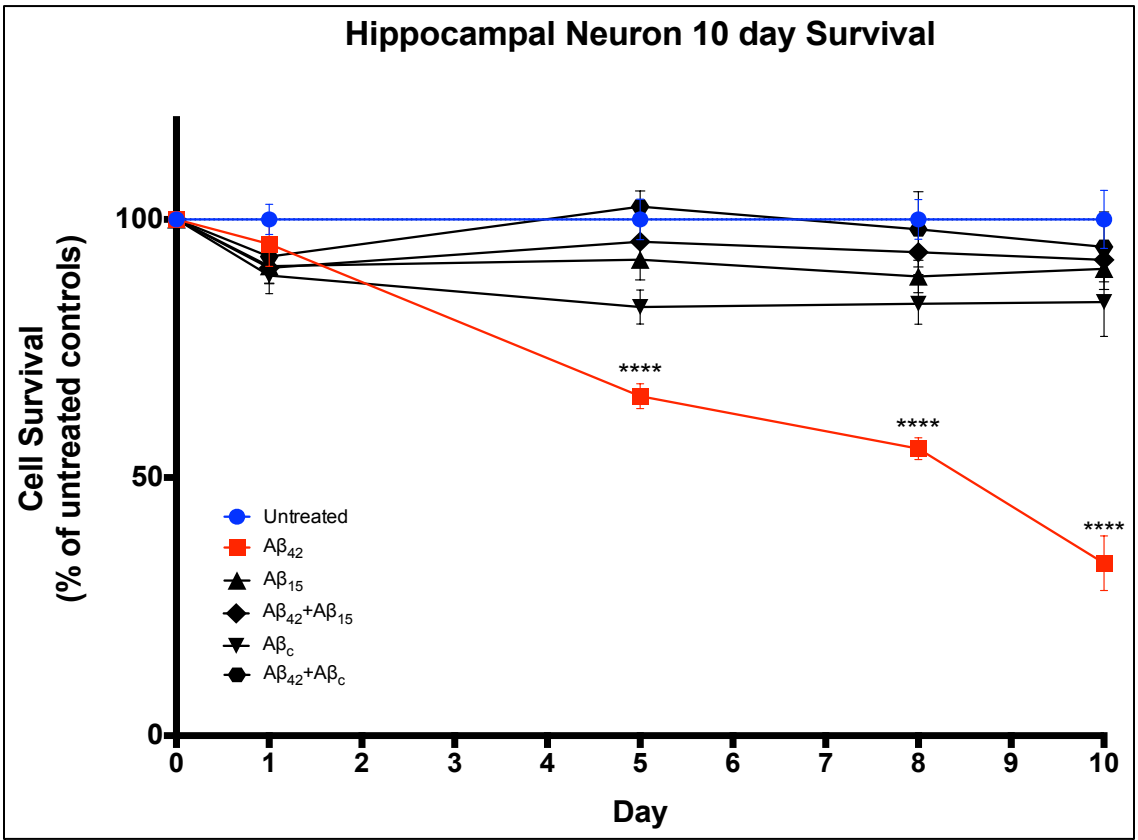

Figure 18. **The N-A β fragment and the N-A β core protect against A β_{1-42} -induced cell death in primary hippocampal neurons.** Cell counts in primary hippocampal cell cultures treated daily with 1μM A β_{1-42}, N-A β fragment or N-A β core alone, or combination daily treatments with A β_{1-42} and N-A β fragment or N-A β core for 10 days (n=4 experiments/group from 3 random fields of view with 2 replicates per experiment). Averaged data are means ± SEM, *p<0.05, **p<0.001, ***p<0.0001 (Tukey *post hoc* tests).

Discussion

Due to its short, hydrophilic nature, as well as its strong agonist-like action via nAChRs, $A\beta_{1-15}$ is an endogenous peptide that was speculated to have neuroprotective function.[2] The current work shows that $A\beta_{1-15}$ is not only non-toxic, but is also neuroprotective in both nAChR-transfected NG108-15 cells as well as primary hippocampal neuronal cultures.

From ROS experiments in NG108-15 cells, nAChRs sensitized cells to $A\beta_{1-42}$ as previously reported.[39] Part of the pathophysiology of AD causing high Aβ deposition is the impairment of the cholinergic nAChR system.[34] Furthermore, in AD brains, the neurons expressing nAChRs are the most vulnerable against Aβ toxicity.[35] Aβ toxicity could partly be mediated by nAChRs, as it triggers signaling cascades such as the MAP kinase-linked signaling pathways, specifically activating ERK and JNK followed by increased PHF-tau leading to increased ROS production and cell death.[57] Our lab has shown these signaling cascades to be activated in NG108-15 cells transfected with α4β2 nAChRs, wherein Aβ toxicity presents within the first day of treatment. Alternatively, there are nAChR-linked neuroprotection pathways. The PI3/AKT pathway is an anti-apoptotic pathway that is well understood in nicotine neuroprotection.[35] This could possibly be the alternative neuroprotective pathway being activated by $A\beta_{1-15}$. It is interesting to see that priming and the $A\beta_{1-15}$ co-treatment (100nM and 500nM) could prevent Aβ-induced ROS production, suggesting that a neuroprotective pathway activated before or within the same timing as full-length Aβ is sufficient enough to prevent activation of ROS-producing or cell-death pathways. Curiously, the dose-

response relationship for $A\beta_{1-15}$ protection against $A\beta_{1-42}$ –induced ROS differed from that seen for nuclear fragmentation. Moreover, the dose-response relationship was quite steep, indicating an unconventional interaction between $A\beta_{1-15}$ and $A\beta_{1-42}$, either at the level of the target receptor(s) and/or downstream signaling.

In contrast, introducing $A\beta_{1-15}$ one day after $A\beta_{42}$ treatment did reduce ROS significantly, but not to baseline levels. This suggests that the Aβ-toxicity pathway had already commenced, and the attenuation by $A\beta_{1-15}$ may have been predominantly mediated by interrupting the toxicity signaling pathways and/or activating anti-apoptotic pathways. These may be primary pathways initiated in the α4β2 nAChR-transfected NG108-15 cell line, and future experiments could be directed toward examining them.

It would not be surprising, however, that there are several other pathways involved in the more physiologically relevant primary hippocampal neuron cultures. In fact, a recent study exploring insulin-mediated protection against Aβ toxicity in primary cultures showed decreased phosphorylation of Akt, and this is prevented by insulin. In contrast, Aβ increased ERK phosphorylation in parallel with apoptosis which was also prevented with inhibition of ERK phosphorylation by insulin.[59] These could be possible pathways targeted by $A\beta_{1-15}$.

In addition, our test of the $A\beta_{1-15}$ and $A\beta_{core}$ against glutamate excitotoxicity showed that only the $A\beta_{1-15}$ at micromolar concentrations was able to partially decrease ROS. These data suggest that the neuroprotection by the N-terminal fragment and the N-terminal core fragment is more selective for Aβ toxicity.

This study clearly shows that $A\beta_{1-15}$ is able to prevent oxidative stress production and nuclear disintegration due to $A\beta_{1-42}$ toxicity in both the model neuroblastoma cell line as well as the more physiologically relevant primary hippocampal cells. This effect is shown in early stages of toxicity (5-day treatments in primary cells) as well as later stages of toxicity (10-day treatments in primary cells), providing us with ample data supporting our hypothesis that $A\beta_{1-15}$ is neuroprotective against $A\beta_{42}$ toxicity in our *in vitro* and *ex-vivo* model systems.

CHAPTER 3

THE NEUROPROTECTIVE ACTION OF $A\beta_{1-15}$ AGAINST $A\beta_{1-42}$-TRIGGERED DEFICITS IN SYNAPTIC PLASTICITY

THE NEUROPROTECTIVE ACTION OF A β_{1-15} AGAINST A β_{1-42}-TRIGGERED DEFICITS IN SYNAPTIC PLASTICITY.

Introduction

Cognitive impairments, especially compromise of short-term memory, are among the first symptoms that present in AD patients. This is due to the synaptic dysfunction triggered by Aβ oligomers involved in the pathogenesis of AD.[25,60] This, in turn, causes impairments in long-term potentiation (LTP), an essential link to memory processing in the hippocampus. Specifically, long-term potentiation is a mechanism by which synaptic strengthening occurs. LTP can be sustained for several hours *in vitro* and several months *in vivo*, demonstrating a physiological change important for learning and memory.[61]

The work produced by Puzzo et al. showed that Aβ is a neuromodulator that has hormetic effects: At low picomolar levels it enhances LTP, but at high nanomolar concentrations it inhibits LTP.[7,9,27] In AD, Aβ is produced in high amounts, leading to deficits in synaptic plasticity and memory. Aβ is also known to regulate several major intracellular pathways that are downstream from NMDA receptor signaling. Key components of these pathways include: calcineurin, CAMKII, cAMP/PKA, protein phosphatase I, and CREB.[62,63] There is growing evidence that suggests a strong association of LTP deficits and Alzheimer's disease, implying LTP impairment could be an early event in AD pathology.[64]

To better understand the possible neuroprotective effects of $A\beta_{1-15}$, we used this information to first, examine whether $A\beta_{1-15}$ had similar hormetic effects on LTP; then, to

test if $A\beta_{1-15}$ can prevent LTP inhibition by $A\beta_{42}$; and finally, to examine whether $A\beta_{1-15}$ could potentially rescue LTP in an AD mouse model known to have synaptic dysfunction.

1. Experimental Design:

Three electrophysiology experiments were performed to examine a variety of effects of $A\beta_{1-15}$, on synaptic plasticity. These are:

1. $A\beta_{1-15}$ dosage effect on synaptic plasticity-

   According to our previous findings with confocal calcium imaging, $A\beta_{1-15}$ is highly potent and active via nAChRs.[2] Dosage effects of AS_{1-15} on synaptic plasticity are therefore important to consider. WT hippocampal brain slices were perfused with either aCSF alone, or 50nM, 50pM or 50fM $A\beta_{1-15}$, for 20 minutes before theta-burst stimulation (TBS) and field potentials (fEPSPs) were recorded for an hour to observe dosage effects on LTP.

2. Inhibition of LTP by $A\beta_{1-42}$ and potential reversal by $A\beta_{1-15}$ –

   Hormetic effects of $A\beta_{1-42}$ have been clearly shown by Puzzo et al., with high nM levels blocking LTP. If $A\beta_{1-15}$ is neuroprotective, it should be able to reverse this block. To demonstrate this, we first perfused WT slices with 500nM $A\beta_{1-42}$, to confirm blocking of normal high-frequency stimulation (HFS) LTP by full-length Aβ. After this was confirmed, we proceeded with a priming experiment in which slices were perfused with $A\beta_{1-15}$ for 20 minutes, followed by perfusion of $A\beta_{1-42}$ for another 20 minutes, followed by HFS. Responses were recorded for an hour to investigate the long-term effect of the priming of synapses with $A\beta_{1-15}$.

3. LTP rescue by $A\beta_{1-15}$ in APPswe mice-

APPswe mice carry a transgene for APP with the Swedish mutation (APP_{695} with double mutation K670N and M671L, Taconic). They have been shown to exhibit LTP deficits beginning at 3-5months of age.[65,66] We hypothesized that $A\beta_{1-15}$ may be able to rescue or reverse LTP deficits in this mouse model of AD. To test this hypothesis, LTP deficits had to be first confirmed in APPswe mice, and this was achieved by perfusing aCSF alone onto the brain slice for 20 minutes, followed by the basic HFS protocol and recording for 1 hour to observe long-term effects. Next, to examine the effect of $A\beta_{1-15}$ on APPswe LTP deficit, the APPswe hippocampal slices were perfused with $A\beta_{1-15}$ for 20 minutes before HFS and responses were recorded for an hour to observe the long-term effects of the $A\beta_{1-15}$ on synaptic plasticity. Controls involved the same treatments on brain slices from wild-type littermates.

2. Materials and Methods

Animals:

Electrophysiology was performed on hippocampal slice cultures prepared from 2- to 5-month-old C57BL/6J mice (Jackson Laboratory) or 5- to 6-month-old APPswe and B6.SJL (background control) mice (Taconic Biosciences). All animal studies were carried out following approved IACUC protocols in accordance with animal welfare guidelines.

Acute Slice preparation:

Swift cervical dislocation and decapitation was performed on the mice (as per an approved IACUC protocol). The brains were extracted and transversely sliced at 350μm using a Leica vibrating microtome (Leica, VT1200S) in artificial cerebrospinal fluid (aCSF) consisting of: 130mM NaCl, 3.5 mM KCl, 10mM glucose, 1.25mM NaH_2PO_4, 2.0mM $CaCl_2$, 1.5mM $MgSO_4$, and 24mM $NaHCO_3$, equilibrated at pH 7.4. The hippocampi were then dissected out using a dissecting microscope, the slices were incubated for 30mins in 95% O_2/5% CO_2 in aCSF at room temperature, then transferred into a 32°C water bath for another 30-min incubation before electrophysiological recording.[2]

Electrophysiology:

Slices were stimulated at the Schaffer collaterals at 0.1Hz with 3V using a bipolar stimulating electrode and recorded from the CA1 pyramidal cells with a glass electrode

filled with 3M NaCl. Stimulation was set to 20-40% of maximum response via two different stimulation protocols. LTP data are presented as averaged field EPSP slope per minute.

1. Theta-burst stimulation (trains of four pulses at 100 Hz, with 10 trains delivered at 5 Hz, each repeated three times every 15 s (three bursts)) or
2. High frequency stimulation (two single trains of 100 Hz separated by 20 s) after perfusion of different concentrations of either $A\beta_{1-42}$, or $A\beta_{1-15}$ was applied as described in Lawrence, Tong, et al, 2014.[2]

Statistics:

ANOVA with Bonferroni post hoc test was used to compare conditions (last 10 minutes for LTP). At least 4 biological replicates were performed for each study. The minimum criteria for significance was $p<0.05$. All data were plotted ± SD.

3. Results:

We initially tested whether the potential impact of $A\beta_{1-15}$ on LTP would be dependent on the timing of perfusion (before or after HFS). Preliminary tests on WT hippocampal slices showed the treatment media had to be perfused before HFS to see the LTP effect, as no effected was seen with treatment perfused after HFS (Fig. 19A). It was important to establish a stable baseline that does not drift to make conclusions about sustained LTP, so we tested the effect of $A\beta_{1-15}$ on baseline synaptic activity. The perfusion of different concentrations of $A\beta_{1-15}$ (100pM and 100nM) did not significantly alter baseline synaptic strength (Fig. 19B).

Wild-type hippocampal slices were subsequently perfused with different concentrations of $A\beta_{1-15}$ and basic LTP experiments were performed using theta-burst stimulation. We initially tested for significant differences in baseline synaptic strength that could perhaps affect our results. We assessed this by measuring fESPS versus stimulus intensity, and plotting input/output curves with increasing stimulation, showing no differences between slices (Fig 20A). We compared theta-burst potentiation and saw a significant difference in early potentiation in slices perfused with femptomolar but not picomolar concentrations of $A\beta_{1-15}$ (Fig. 20B, $p<0.05$). Surprisingly, the $A\beta_{1-15}$ fragment was able to sustain enhanced LTP at a concentration of 50fM (Fig. 20C & D, $p<0.05$), an unexpectedly high potency.

We then directly examined the effect of $A\beta_{1-15}$ on $A\beta_{1-42}$-treated slices. High-frequency stimulation LTP was substantially attenuated by 100nM $A\beta_{1-42}$ perfused into the slice for 20 minutes before stimulation (Fig. 20E & F, $p<0.05$), as previously

reported.[7] Pre-incubation of the brain slice with $A\beta_{1-15}$ for 20 minutes before full length Aβ perfusion was able to prevent this LTP block by $A\beta_{1-42}$, restoring LTP to control levels (Fig. 20E & F, $p<0.05$).

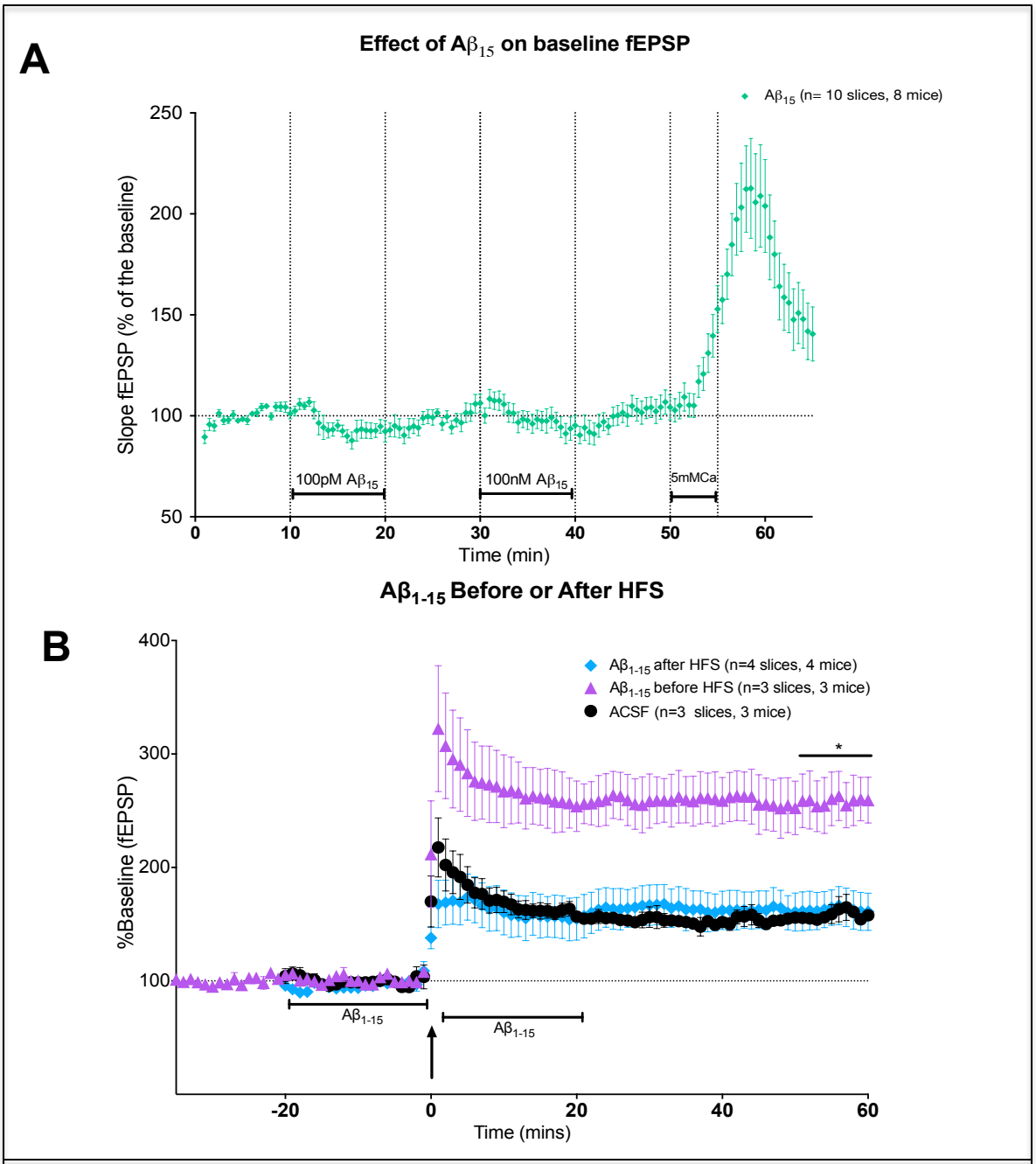

Figure 19. **The N-terminal A β_{1-15} has no significant effect on baseline synaptic activity and has to be perfused before HFS to have an effect on LTP**. Effect of A β_{1-15} perfusion in WT brain slices: A. on baseline synaptic activity showing no significant baseline drift with A β_{1-15} perfusion at 100pM or 100nM concentrations. 5mM calcium used as a positive control B. before or after HFS showing perfusion of treatment media before HFS is required for LTP enhancement. on baseline synaptic activity showing no significant baseline drift with A β_{1-15} perfusion at 100pM or 100nM concentrations. 5mM calcium used as a positive control. Data plotted are means ± SD (*p<0.05)

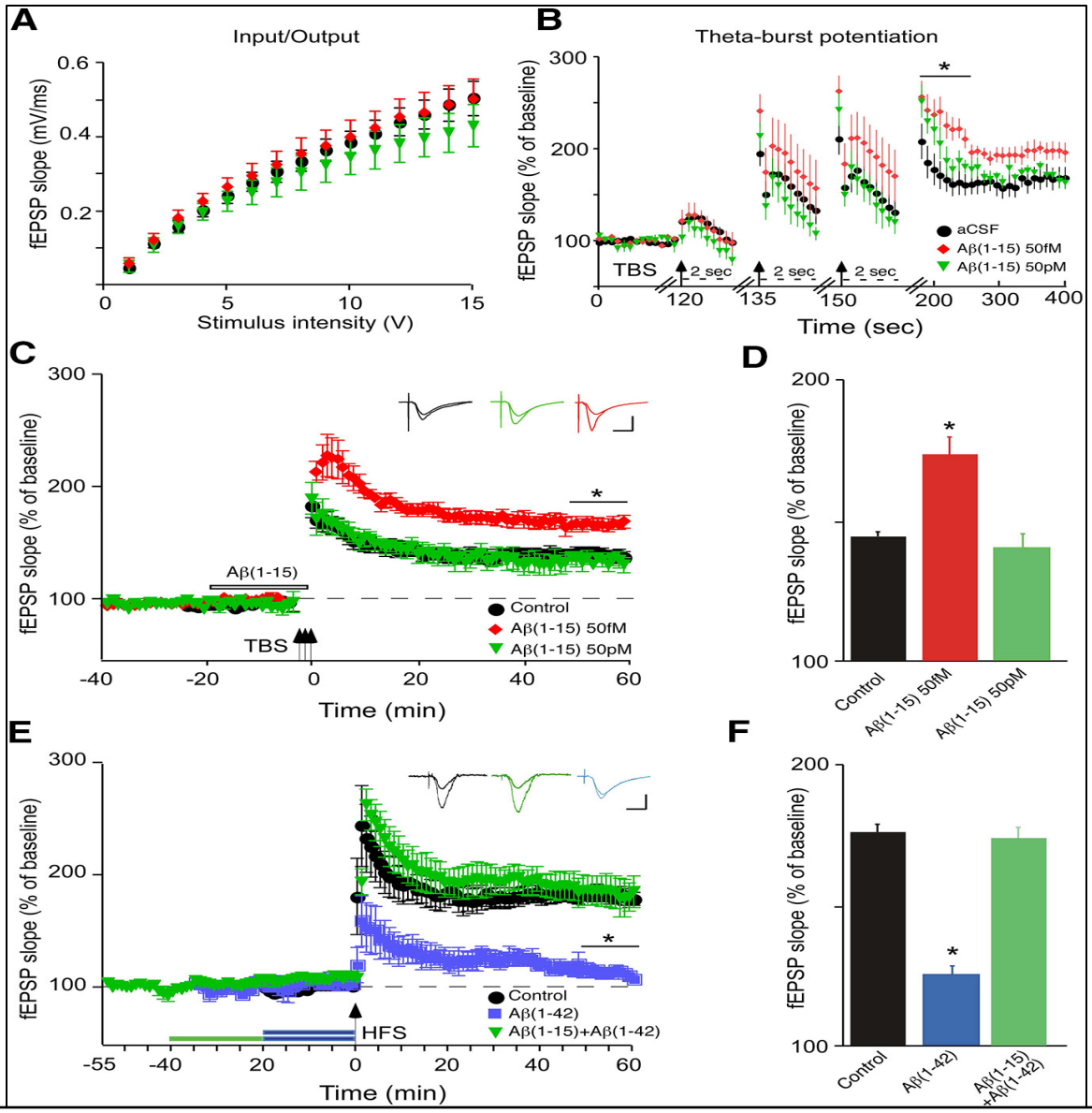

Figure 20. **A β $_{1\text{-}15}$ enhances LTP at femptomolar concentrations and can prevent LTP block by A β $_{1\text{-}42}$.** A-D. LTP experiments with TBS in presence or absence of A β $_{1\text{-}15}$ in WT slices. A. Control input/output curves, before treatment. B & C. 50fM A β $_{1\text{-}15}$ can significantly increase PTP and LTP. Color coded insets showing control fEPSPs for control aCSF (black), 50pM $A\beta_{1\text{-}15}$ (green), and 50fM $A\beta_{1\text{-}15}$ (red) D. Averaged fEPSP values for final 10 minutes of LTP showing significantly enhanced LTP in WT slices treated with $A\beta_{1\text{-}15}$.
E & F. LTP experiments with HFS in absence or presence of A β $_{1\text{-}42}$ with or without pretreatment with A β $_{1\text{-}15}$ in WT slices showing rescued LTP when pre-treated with 500nM $A\beta_{1\text{-}15}$ compared to LTP blocked by $A\beta_{1\text{-}42}$ alone. Color coded insets showing fEPSPs for control aCSF (black), 500nM $A\beta_{1\text{-}15}$ (green), and 500nM $A\beta_{1\text{-}42}$ (blue) F. Averaged fEPSPs for last 10 minutes of LTP as noted by solid black bar in E representing LTP rescue by $A\beta_{1\text{-}15}$. *n*= 6 slices/ group. Data plotted are the means ± SD *p<0.05 (Bonferroni *post hoc* tests).

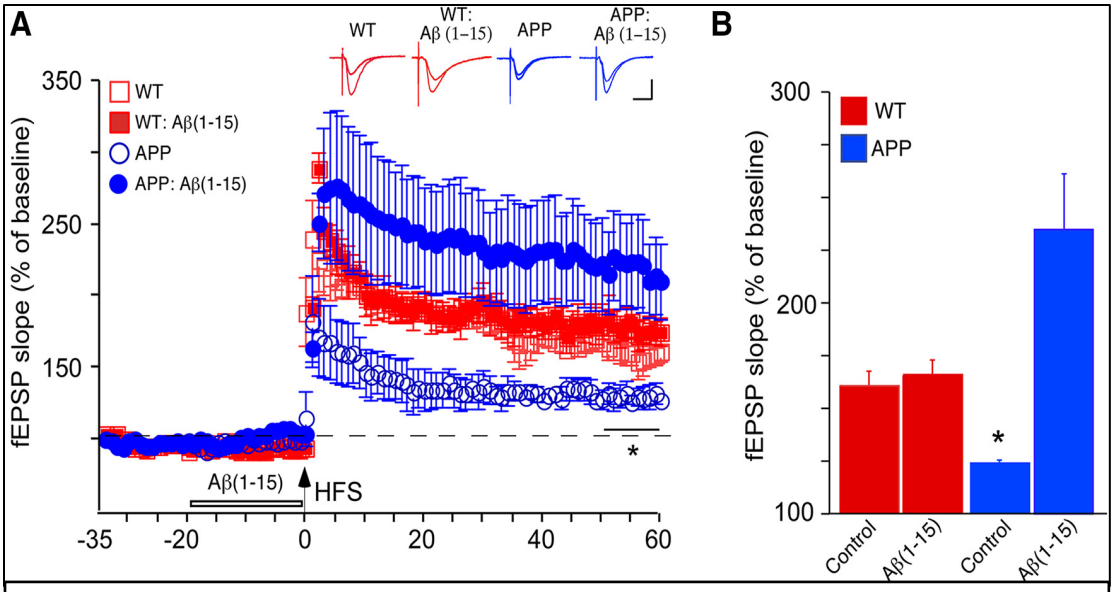

Figure 21. **A β $_{1\text{-}15}$ restores normal LTP in APPswe mouse hippocampal slice.** A. HFS-induced LTP with color-coded insets showing example fEPSPs for slices from WT mice with or without pretreatments with 500nM A β $_{1\text{-}15}$ (red) or slices from APPswe mice with or without pretreatment with 500nM A β $_{1\text{-}15}$. Period of $A\beta_{1\text{-}15}$ pre-treatment marked with an open bar. B. Average fEPSP slope values for the last 10 minutes of LTP as noted by the solid bar in A. n= 4slices/group. Data plotted are the means ± SD *p<0.05 (Bonferroni *post hoc* tests).

Lastly, APPswe mouse acute hippocampal slices were used in HFS LTP experiments. First, when the WT slices were perfused with $A\beta_{1\text{-}15}$ before HFS, we saw no differences between slices compared to aCSF alone. In contrast, APPswe mice are known to have HFS LTP deficits, and this was confirmed by perfusing aCSF alone and performing basic LTP (Fig. 21A & B, $p<0.05$). APPswe slices that were perfused with $A\beta_{1\text{-}15}$ before HFS, showed this deficit was eliminated (Fig. 21A & B, $p<0.05$). These data support our hypotheses that $A\beta_{1\text{-}15}$ can both rescue LTP and protect against the effects of $A\beta_{1\text{-}42}$, here produced endogenously in the transgenic APPswe.

Discussion:

It is well established in the literature that Aβ oligomers disrupt synaptic signaling via interaction with various receptors perturbing various signaling pathways (Fig. 22) (reviewed in ref 1). While the molecular mechanism is not well understood, it is generally believed that high concentrations of Aβ oligomers trigger overstimulation via multiple pathways, causing abnormal redox reactions and calcium upregulation. This is followed by the activation of downstream pathways that cause a cascade of pathological events including the production of high oxidative stress, mitochondrial dysfunction, synaptic dysfunction and neuronal loss.[39,64,67,68]

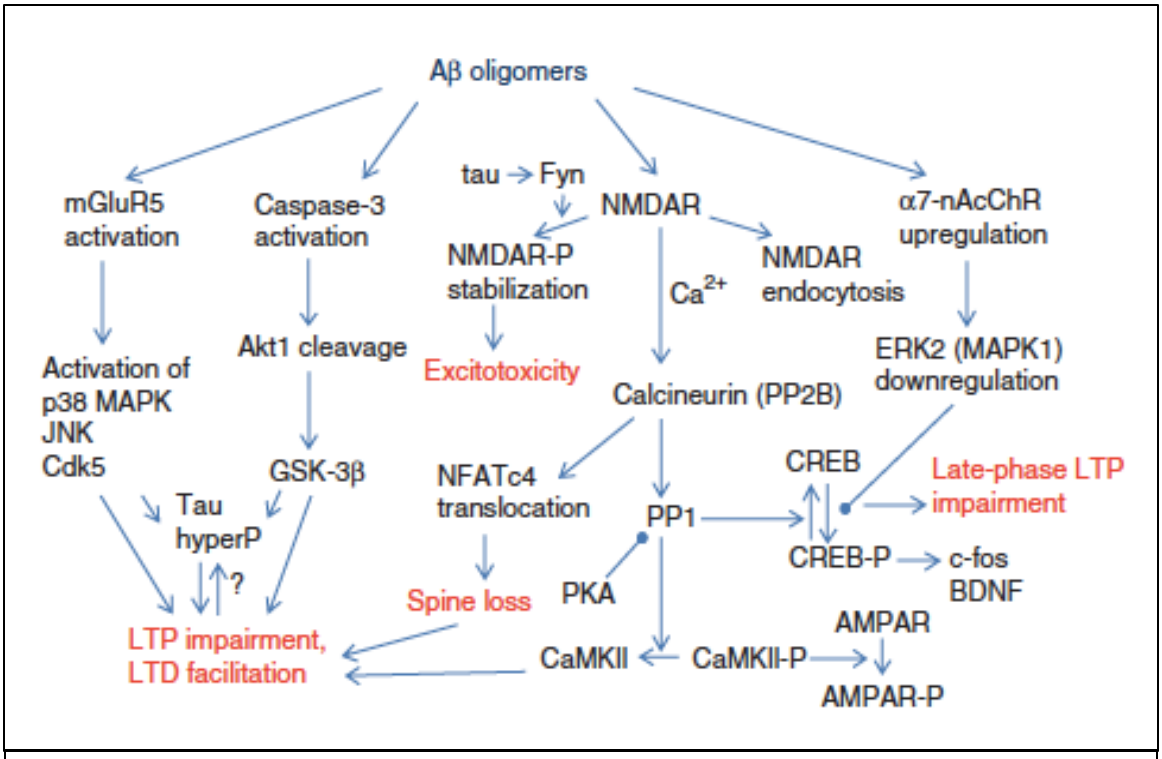

Figure 22. **Proposed molecular mechanisms of A β synaptotoxicity.**[1]

In this study we observed that, comparable to full-length Aβ, $A\beta_{1-15}$ was able to enhance LTP at low concentrations when it was perfused before TBS, further confirming its neuromodulatory function. It would be interesting to examine possible hormetic effects as reported by Puzzo et al. (2010), wherein full length Aβ was shown to enhance LTP at low pM concentrations but block LTP at higher nanomolar concentrations.[7] This would be rather unlikely due to the short, hydrophilic nature of $A\beta_{1-15}$ and the fact that at picomolar levels it had no effect on TBS LTP (Fig.20, A-D).

It is surprising to see that in our experiments, $A\beta_{1-15}$ was completely able to reverse LTP deficits induced by $A\beta_{1-42}$. The mechanism by which this was achieved is currently unknown due to the large number of intracellular pathways with which $A\beta_{42}$ oligomers can interact. However, we can conjecture from the known LTP mechanism that there could be a rapid upregulation of AMPA receptor trafficking to the synapse.[38,40,62] It could also simply be that $A\beta_{1-15}$ is blocking the binding of $A\beta_{42}$ to its target receptors, thus blocking its deleterious effects and allowing recovery to the 'normal' state. The latter is unlikely to be the only mechanism acting, due to the ability of $A\beta_{1-15}$ to rescue memory in APPswe mice in which elevated levels of toxic Aβ are already present for weeks to months.

Similar studies exploring synaptic plasticity showed that pre-treatment of slices with Neurotrophin 4 rescued LTP impairment caused by Aβ-induced deficits that resulted in impaired CAMKII signaling pathways.[69] This study was focused on CAMKII downstream from the NMDA receptor and the phosphorylation of AMPA receptors dependent on CAMKII. BDNF and Neurotrophin 4 were both able to enhance CAMKII

and AMPAR phosphorylation and compete against Aβ inhibition to enhance LTP in co-treated slices.[69] While this was an exciting study, it focused solely on CAMKII and AMPA receptors. However, it would be interesting to investigate the impact of $A\beta_{1-15}$ on neurotrophins.

A similar study explored the effect of resveratrol on Aβ toxicity. The authors were able to show that resveratrol prevented LTP impairment *in vivo* in correlation with rescued CREB activation that was also inhibited by Aβ.[70] Resveratrol is able to rescue CREB activation, which is able to bind BDNF promoters, which, in turn, increases AMPA receptor activity.[71]

It would be interesting assess whether $A\beta_{1-15}$ has similar effects on these specific receptors and downstream signaling pathways (explored in Chapter 4), but also important to understand how $A\beta_{1-15}$ is interacting with other pathways, or if it is just blocking the effects of $A\beta_{1-42.}$

While this study shows significant effects of the $A\beta_{1-15}$ fragment on LTP, more in-depth electrophysiology studies exploring effects of $A\beta_{1-15}$ on long-term depression (LTD), which we know is enhanced by $A\beta_{42}$, and sub-threshold LTP, need to be performed and the mechanisms involved should be further explored.

CHAPTER 4

THE NEUROPROTECTIVE ACTION OF $A\beta_{1-15}$ AGAINST $A\beta_{1-42}$-TRIGGERED CHANGES IN FEAR MEMORY AND ANXIETY.

THE NEUROPROTECTIVE ACTION OF $A\beta_{1-15}$ AGAINST $A\beta_{1-42}$-TRIGGERED CHANGES IN FEAR MEMORY AND ANXIETY.

Introduction:

Cognitive decline, including memory loss, is considered an early symptom in AD, but they occur fairly late in the pathophysiology of the disease, often fifteen to twenty years after amyloid accumulation first occurs (Fig. 23).[1] In later stages, AD patients display increased anxiety and agitation.

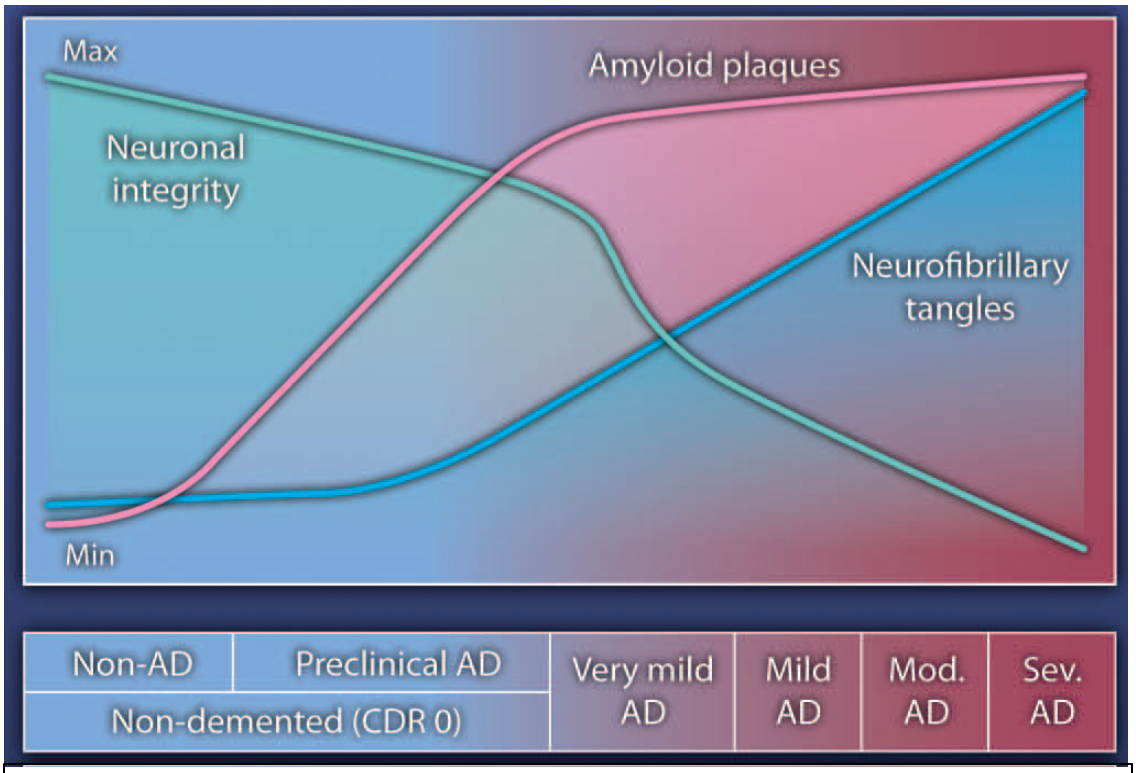

Figure 23. **Timeline of the pathophysiology of AD**[1] Amyloid deposition begins 15-20 years before any cognitive symptoms arise.

The current FDA-approved treatments for AD fall under two categories: Cholinesterase inhibitors and NMDA receptor antagonists. Both are able to lessen

severity of symptoms for a limited amount of time but not cure the disease.[24,72] This makes it imperative to explore new avenues for AD therapies.

Amyloid-β oligomers are responsible for perturbing various functions within and between neurons. They disrupt mitochondrial function, cholinergic signaling, and overall calcium homeostasis, while also activating stress pathways.[35,56,73] As previously noted, in relation to learning and memory, we know Aβ binds synapses in the hippocampus, specifically excitatory synapses that express NMDA receptor subtypes NR1 and NR2B.[68] There is also evidence showing that Aβ interacts with several other receptors affecting intracellular pathways downstream of receptor signaling (see Fig. 22), which in turn, translate to memory deficits.[62]

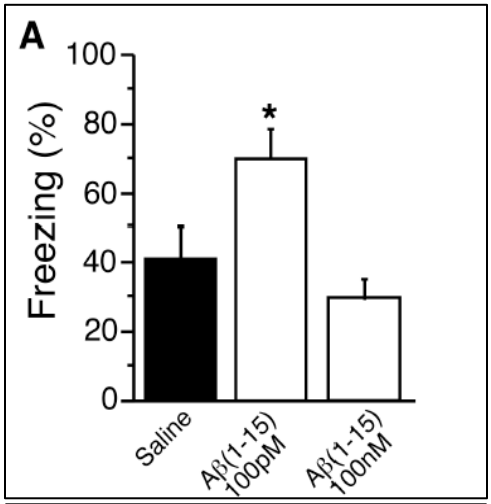

Figure 24. **At 100pM, A β_{1-15} enhances memory in WT mice.** Bilateral injection of 100pM, 100nM $A\beta_{1-15}$, or saline into the dorsal hippocampus 24 hours before single trial CFC testing. Freezing was measured via TSE videotracking software. Data are means ± SEM *p<0.05 [3]

To best understand the phenotypic alterations caused by Aβ toxicity and the possibility of preventing those alterations using the N-terminal $A\beta_{1-15}$ fragment , we explored several behavior paradigms in a well-established AD mouse model, 5XFAD, to examine learning deficits affected by Aβ accumulation in different parts of the brain. The earliest and most severe alterations occur in the hippocampus and entorhinal cortex of the medial temporal lobe.[55] These areas will be the focus of these tests. The three behavior tests we used to focus on these regions were: (1) Contextual fear conditioning (CFC), exploring

contextual and fear memory; (2) Novel object recognition (NOR), examining recognition memory; and finally, (3) the Elevated plus maze (EPM), to observe the effect of the N-terminal Aβ fragment on anxiety levels. The rationale for exploring the potential for N-terminal Aβ fragment protection against Aβ-linked behavioral deficits in the AD mouse model was based in the demonstration that $A\beta_{1-15}$ enhanced CFC in wild-type mice (Fig. 24), further supporting the neuromodulatory role for the N-terminal Aβ fragment.

In coordination with memory tests we also tested for changes (compared to WT saline injected mice) in the NMDA (N-methyl-*D*-aspartate)-type and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-type glutamate receptor subunits, which are linked to memory processing. We also tested for changes in activated JNK (c-Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase), which are linked both to memory and neurotoxicity. Finally, we investigated CREB (cAMP response element binding protein) signaling, which plays a central role in memory. The overall goal for this study was to investigate whether $A\beta_{1-15}$ improves the AD phenotype *in vivo* and elucidate the signaling mechanisms involved.

1. Experimental Design:

Learning and memory deficits are the first symptoms to appear in AD, and these deficits progress rapidly, disrupting patients' lives. These consequences underscore the importance of studying the effects of Aβ on memory, and exploring ways to enhance memory or reduce memory deficits in AD. To parallel the electrophysiology studies and investigate memory deficit reversal in an AD mouse model, we performed various behavioral assays.

5XFAD mouse model:

The 5XFAD mouse model (MMRC Stock # 34840-JAX, Tg 6799: B6SJL-Tg(APPSwFlLOn,PSEN1*M146L*L286V) was found to be best suited for the behavioral experiments due to its accelerated AD pathology leading to early plaque deposition, neuron loss and memory deficits.[74] This AD mouse model is a transgenic multiple APP/Presinilin 1 (PS1) model expressing five FAD mutations leading to high Aβ production and deposition. Like many AD mouse models, it lacks neurofibrillary tangle formation. Regardless, the age-dependent neurological deficits it displays resemble key common phenotypes in AD patients, particularly with regard to Aβ-linked pathology, making it an ideal model for use in behavior testing.

Contextual Fear Conditioning (CFC):

Fear conditioning tasks are well-established tests that examine associative memory affected in AD patients. The brain regions involved in FC are the amygdala and the

hippocampus. The hippocampus is involved in the learning of the context that triggers fear. The amygdala, on other hand, is a brain structure highly involved in the fear circuit. Therefore, the hippocampus acts as a sensory relay communicating the context to the amygdala about the environment of the dangerous event.

1. First, we aimed to confirm that the 5XFAD mice did indeed display memory deficits compared to their wild-type counterparts in our experimental system. Behavior experiments (such contextual/cued fear conditioning, elevated plus maze, and novel object recognition) were performed on 5XFAD and WT mice at 6-7 months of age (when memory deficits begin to appear)[75–77]. Once the conditions were established that revealed memory deficits in 5XFAD mice compared to WT mice, we were able to move on to rescue experiments.
2. Memory rescue: 5XFAD mice have been shown to have memory deficits in CFC, making them an ideal AD model to test $A\beta_{1\text{-}15}$-induced memory rescue.[78, 79] Based on our dosage effects from previous experiments[8], we used the most effective $A\beta_{1\text{-}15}$ concentration (500nM or control saline) to inject into the dorsal hippocampus of 5XFAD mice and then train and test in CFC and other behavior paradigms to see whether $A\beta_{1\text{-}15}$ can rescue memory deficits in the 5XFAD mice. It would be very significant to be able to show a memory rescue *in vivo* in an established AD mouse model and would further support our hypothesis of $A\beta_{1\text{-}15}$ being neuroprotective.

Novel Object Recognition (NOR):

Impairment of recognition memory is part of declarative memory deficits that present in AD patients. Novel object recognition tests evaluate recognition memory using a simple task with no outside reinforcement, thereby testing a rodent's natural preference for novel objects. The brain regions involved in NOR are the hippocampus and the perirhinal cortex. The perirhinal cortex is necessary for representation of basic information of whether an object is familiar or novel. The hippocampus is important for encoding data linked to that basic information connected to the experience within the object-related context.[80]

Elevated Plus Maze (EPM):

The elevated plus maze (EPM) is a well-known test of unconditioned anxiety. It takes advantage of the rodent's natural preference for a protected, dark area.[81] The brain regions involved in EPM are the amygdala, hippocampus, and cingulate and prefrontal cortices.[81,82]

Protein Extraction and Western Blot:

To begin to elucidate the mechanisms by which the N-terminal peptide can cause behavioral changes in the 5XFAD mouse model, we extracted hippocampal proteins and preliminarily assessed molecular level changes involved in learning and memory as well as stress pathways known to be activated by the toxic full length Aβ.

2. Materials and Methods

Animals:

For each of the behavior experiments, 6.5-8-month old 5XFAD mice or age-matched wild-type B6.SJL mice of roughly equal numbers of either sex were used (Jackson Laboratories). All animal use was followed with approved IACUC protocols in accordance with animal welfare guidelines.

Stereotaxic Cannulation Surgery:

In collaboration with Drs. Cedomir Todorovic and Tessi Sherrin, contextual fear conditioning was utilized to test learning and memory in WT and 5XFAD mice after bilateral microinjection over 30s with saline, 500nM $A\beta_{1-15}$, or 500nM $A\beta_{10-15}$ in a maximum volume of 0.40μL injected into each side. Stereotaxic cannulae implantation was implemented as described (Sherrin et al., 2010). 5XFAD or age-matched B6.SJL mice were fully anesthetized with 1.2% Avertin and cannulae inserted using the following stereotaxic coordinates relative to Bregma: -1.5mm anteroposterior, ±1mm lateral, and -2mm depth into the dorsal hippocampus. After 7 days of recovery the dorsal hippocampi were bilaterally injected with either $A\beta_{1-15}$, $A\beta_{10-15}$, or saline before single-trial CFC, or NOR were performed. Cannula placement was checked in randomly assigned mice after behavior experiments by dissection and transverse slicing of the brain to look

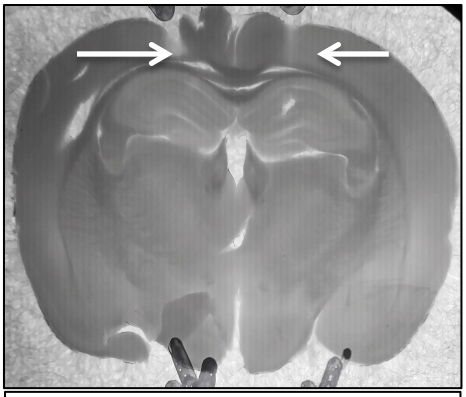

Figure 25. **Cannula placement confirmation**. Cannula placement confirmed by transverse slicing of brain to identify cannula tracks into the dorsal hippocampi (arrows).

at cannula tracks (Fig. 25).

Contextual Fear Conditioning:

Single-trial Contextual Fear Conditioning (CFC) consists of a 180 second exposure to a context that consisted of a fear conditioning box containing a plexiglass cage (36 x 21x 20cm) where the mouse explores the context, followed by a mild foot shock of 0.8mA for 2 seconds delivered through a stainless-steel grid floor. Contextual fear memory recall was tested after 24 hours by measuring freezing response (absence of movement) when the mouse was placed back in the conditioning context. In an subsequent experiment testing remote memory, a test was performed wherein the conditioned mouse was re-introduced to the context 12 days after initial training. Basic locomotion and shock were automatically measured and set by using TSE systems computer-controlled fear conditioning software (TSE, Bad Homburg, Germany). Freezing, which is defined as a lack of movement, was recorded every 10 s for a total of 18 sampling intervals (180 s total) by an observer that was blinded to treatments. A minimum sample size of 6 per group were used for all $A\beta_{1\text{-}15}$ experiments, or 3-4 per group for preliminary $A\beta_{10\text{-}15}$ experiments. Data were graphed and analyzed using one-way ANOVA on Prism (GraphPad).

Novel Object Recognition:

NOR consisted of 3 phases over three days: Habituation phase, Familiarization phase, and Testing phase. The Habituation phase was simply a 10-min open field phase in

which the mice were able to explore the open field box where the NOR test would later be performed. Total distance traveled and time spent in the center of the open field were recorded to ensure similar activity between all groups as well as looking at differences in thigmotaxis between groups. The Familiarization phase was performed 24 hours after the Habituation phase and it consisted of the reintroduction of the mice into the open field, but this time with 2 identical objects placed in opposite corners of the open field box approximately 40cm from the outer walls. In this phase, the time spent with each object as well as the number of visits to each object was recorded. The third and final phase was the Testing phase in which one of the 2 identical objects was replaced with a novel object that was different in shape and color but roughly similar in size. For the final phase the total time spent with each object and total visits per object were recorded. All animal behavior was recorded by a camera connected to a PC and analyzed by video tracking software (VideoMot 2, TSE Systems). For all phases, total distance traveled was recorded to confirm similar activity between all groups. The percentage of time spent exploring novel vs. familiar objects and a discrimination index were plotted. A minimum sample size of 7 per group were used for 5XFAD groups, or 2-3 per group for WT. Data were graphed and analyzed using one-way ANOVA on Prism (GraphPad).

Elevated Plus Maze:

The elevated plus maze is made of four arms that radiate from a central platform with two opposing closed arms with tall dark walls and two opposing open arms forming a

plus shape, elevated 1 meter from the floor. Mice were placed in the central platform facing an open arm and allowed to explore for 5 minutes. All animal behavior was recorded by a camera connected to a PC and analyzed by video tracking software (VideoMot 2, TSE Systems). Recorded data include: Total distance traveled, time spent in open/closed arms, and visits per closed/open arm. Percent of time spent in open arms as well as discrimination ratios were plotted. A minimum sample size of 7 per group were used for 5XFAD groups, or 2-3 per group for WT. Data were graphed and analyzed using one-way ANOVA on Prism (GraphPad).

Protein Extraction:

Hippocampi were rapidly dissected from brains removed from animals 24hrs after Aβ injection and then flash-frozen in liquid nitrogen. Proteins from full hippocampi were extracted by homogenizing the tissue in Pierce lysis buffer (Thermo Scientific, cat#87788) with protease/phosphatase inhibitors, then gently rotated at 4°C for 20 minutes followed by centrifugation at 18,000g at 4°C for 20 minutes. The supernatant was saved and protein concentration was measured using a BCA kit. Supernatant from this lysis / homogenization extraction was equivalent to the cell cytosol.

Extracting compartment proteins was achieved using a compartmental protein extraction kit (Millipore, cat#2145). Briefly, each compartment extraction was achieved by a series of washes with different HEPES buffers and centrifugation. Four fractions were obtained: cytoplasmic, nuclear, membrane and cytoskeletal. These were validated by immunoblotting against different protein markers to their expected compartmental

fractions. Again, protein concentrations for each sample were measured using a BCA kit.

Western Blot:

For western blot, the protein samples were diluted with the buffer used for lysis and loading buffer (Laemmli buffer and β-mercaptoethanol) in a 1:1 ratio. The samples were heated at 95°C on a heating block for 10 minutes, cooled on ice, and then centrifuged. Equal amounts of protein were then loaded on 4-20% Tris-glycine gels and electrophoresed at 125V for 1.5 hours. Proteins were transferred to activated PVDF membranes via iBlot semi-dry system. The membranes were quickly washed with PBS, and then blocked with LI-COR TBS Blocking buffer for 2 hours before incubating overnight with primary antibodies (NR1: Millipore Cat# 05-432, GluR2: Santa Cruz Cat# sc-7611, pCREB: Cell Signaling Cat# 9198). The membranes were washed three times in TBS solution with 0.01% Tween, then incubated with LI-COR IR-detectable secondary antibodies for one hour at room temperature. Finally, the membranes were washed again with TBS Tween, followed by PBS, and imaged using the Odyssey Infrared Imaging system (LI-COR Biosciences). Bands were compared with protein loading standards.

3. Results

Contextual fear conditioning:

We were able to confirm that there is a significant memory deficit in 7-month old 5XFAD mice compared to their age-matched WT counterparts, using single-trial CFC (see Fig. 26 inset from ref 7) to measure baseline freezing responses (Fig. 26, $p<0.05$). We also confirmed no significant differences in males vs. females (not shown).

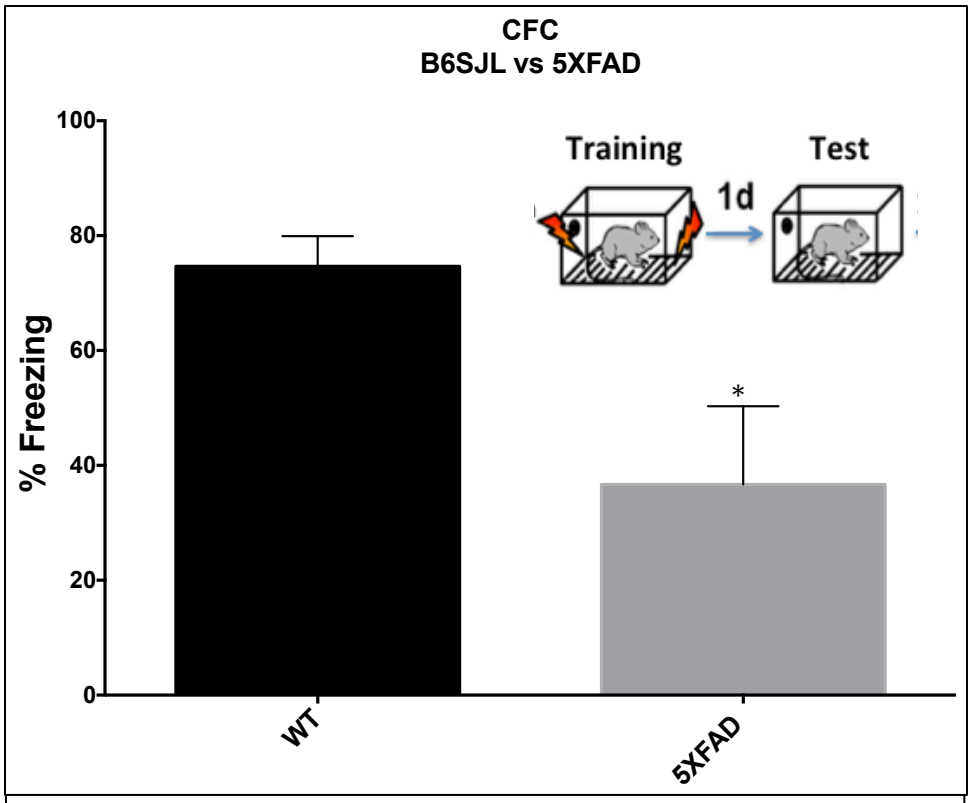

Figure 26. **5XFAD mice exhibit CFC deficit at 7 months of age.** Basic single-trial CFC testing differences between B6SJL mice and 5XFAD mice at 7-months of age. Data are means ± SEM, n=6/group *p<0.05 two-tailed t-test. Inset shows the single-trial protocol (adapted from ref 7).

These baseline results allowed us to proceed to rescue experiments wherein 500nM $A\beta_{1-15}$ or saline were microinjected bilateraly (0.40µL/side) into the dorsal hippocampi of 5XFAD mice just prior to CFC training. First, as expected, the 5XFAD mice injected with saline had significantly lower freezing levels compared to B6.SJL mice injected with saline (Fig. 27, $p<0.05$). The 5XFAD mice injected with $A\beta_{1-15}$ showed freezing levels comparable to WT mice with no memory deficits. This indicates that $A\beta_{1-15}$ was able to rescue (reverse) the 5XFAD mouse memory deficit in comparison to 5XFAD mice injected with saline, which showed very little freezing behavior (Fig. 27, $p<0.005$). No significant differences were observed in males vs. females (not shown).

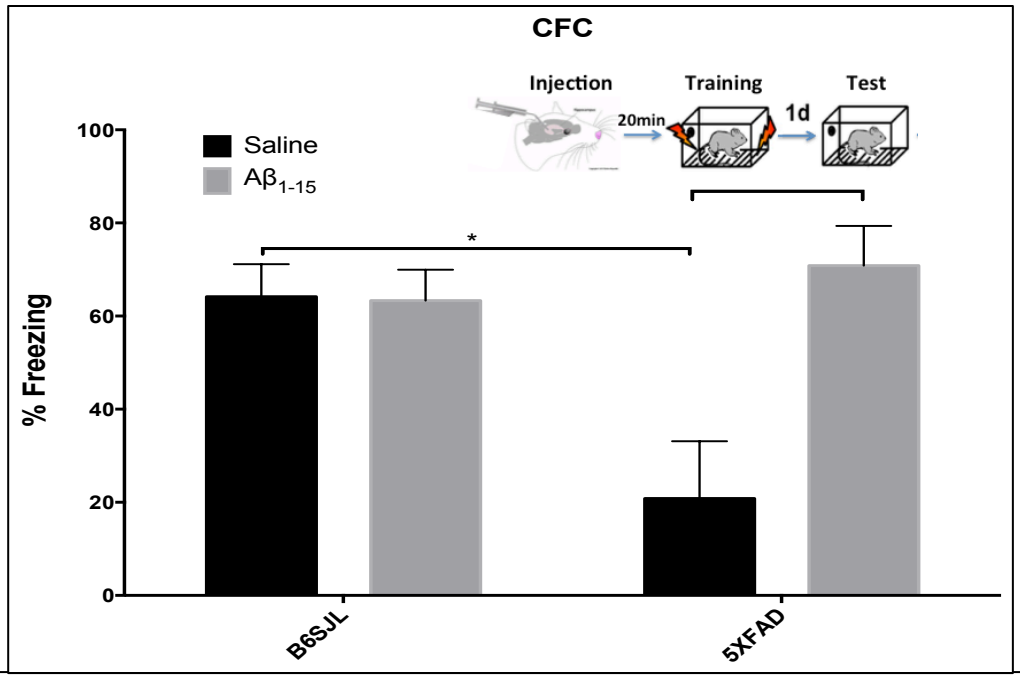

Figure 27. **Bilateral delivery of 500nM Aβ_{1-15} into the dorsal hippocampus rescues memory deficit in 5XFAD mice to normal WT levels.** Mice were trained for CFC single-trial paradigm using a mild foot shock 24h before testing. 500nM Aβ_{1-15} or sterile saline were bilaterally injected into the dorsal hippocampi 20mins before training. Freezing was measured via TSE videotracking software. Conditioned freezing was assessed by two trained observers. (**$p<0.005$ comparing 5XFAD mice injected with $A\beta_{1-15}$ to saline). Data are means ± SEM, n=5-6/group. One way ANOVA with Bonferroni post hoc tests..[7,8]

We repeated the single trial CFC test 24 hours after the initial test, and followed with a remote memory test 12 days later to examine the duration of memory improvement. Mice were placed in the context of shock and observed for freezing for three minutes. Again, the 5XFAD mice injected with $A\beta_{1-15}$ 12 days earlier retained normal levels of freezing that were significantly higher than saline injected 5XFAD mice (Fig. 28, $p<0.05$). For all tests, there was no difference in locomotion or sensory processing between B6.SJL and 5XFAD mice (not shown), confirming that differences observed in CFC were due to altered memory processing.

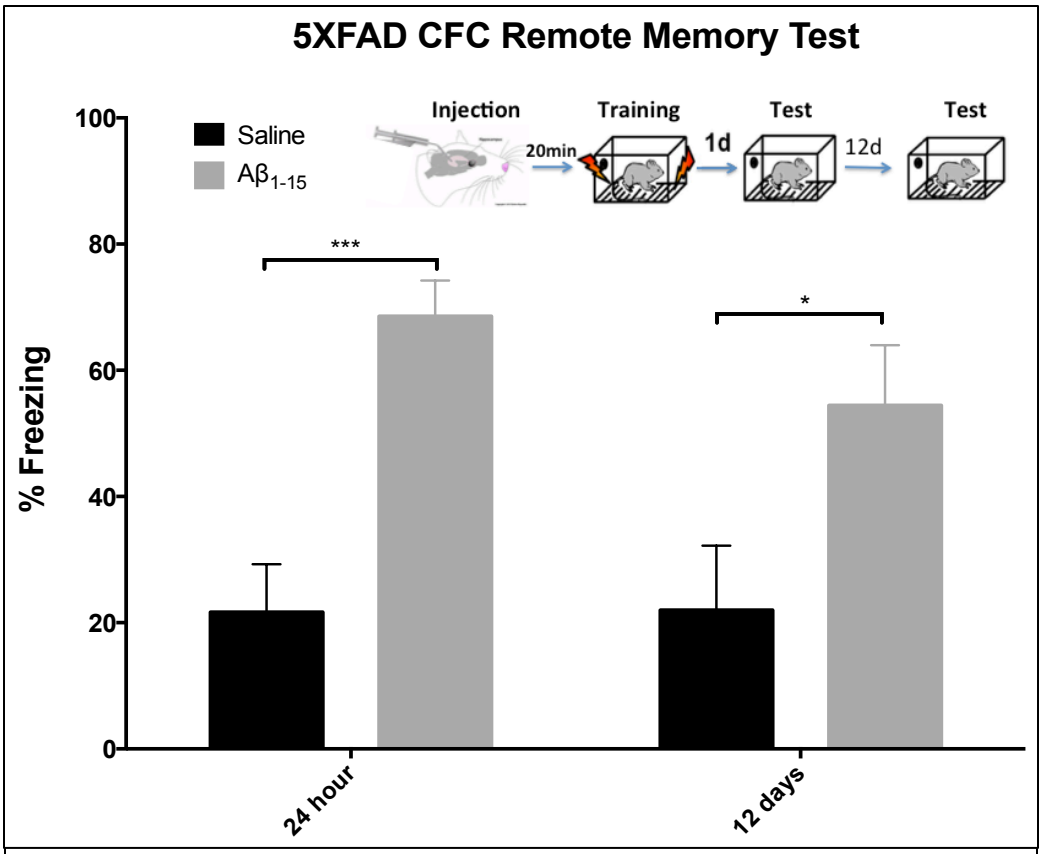

Figure 28. **Bilateral delivery of 500nM $A\beta_{1-15}$ into the dorsal hippocampus rescues memory deficit in both CFC (24h later) and remote memory CFC (12d later) in 5XFAD mice.** Conditions as described in figure 26. CFC (24h) and Remote memory CFC (12d) in 5XFAD mice injected with $A\beta_{1-15}$ or saline controls. Data are means ±_SEM, n=6-7/group *** $p<0.001$, *$p<0.05$ two-tailed t-test[7,8]

To further examine the memory rescue, we tested for any benefits of the core fragment ($A\beta_{10-15}$) in the 5XFAD mice. As the core fragment has been confirmed by our lab to be the active sequence of the $A\beta_{1-15}$ peptide[2], this would further confirm its neuroprotective and memory rescue potential. As expected, $A\beta_{10-15}$ resulted in similar memory rescue as $A\beta_{1-15}$ in both memory tests, 24 hours later and the 12-day remote memory test, with significantly higher freezing measured in groups injected with $A\beta_{10-15}$ than the saline control groups (Fig. 29, $p<0.01$). There was an unusual increase in freezing for the 5XFAD group injected with saline after 12 days, but the freezing response remained significantly reduced compared to the 5XFAD injected with the core fragment.

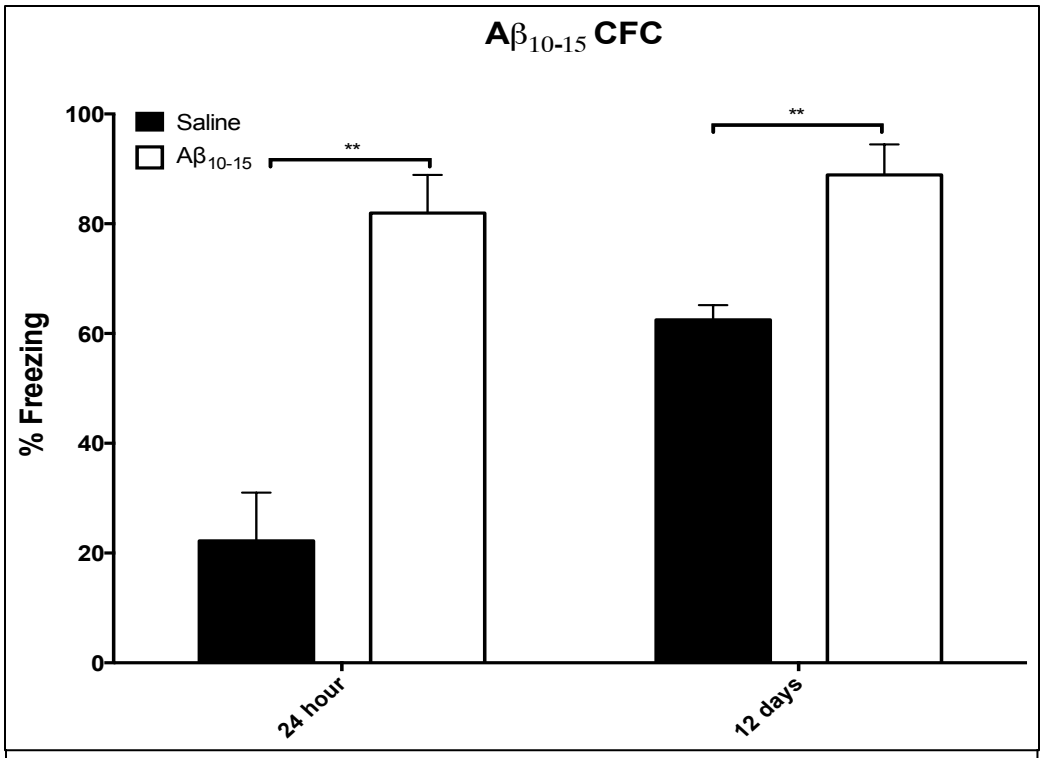

Figure 29. **The N-terminal core fragment A β $_{10-15}$ rescues memory deficit in both CFC (24h later) and remote memory CFC (12d later) in 5XFAD mice.** Training and injection conditions as described in figure 26 with the core fragment instead $A\beta_{1-15}$. CFC and Remote memory CFC in 5XFAD mice injected with A β $_{10-15}$ or saline controls. Data are means ± SEM. n=3-4/group **p<0.01 two-tailed t-test

Novel Object Recognition:

The novel object recognition test was performed to examine whether $A\beta_{1-15}$ is able to rescue recognition memory in the 5XFAD mouse. As part of the 3-day test, we assessed thigmotaxia on the first day (habituation). While differences were not significant between groups in these preliminary experiments, likely due to the smaller sample size (esp. B6.SJL mice), the B6.SJL mice were much less likely to explore the center of the open field than were the 5XFAD mice (Fig. 30), indicating an increase in exploratory behavior by the 5XFAD mice and/or a decrease in anxiety.

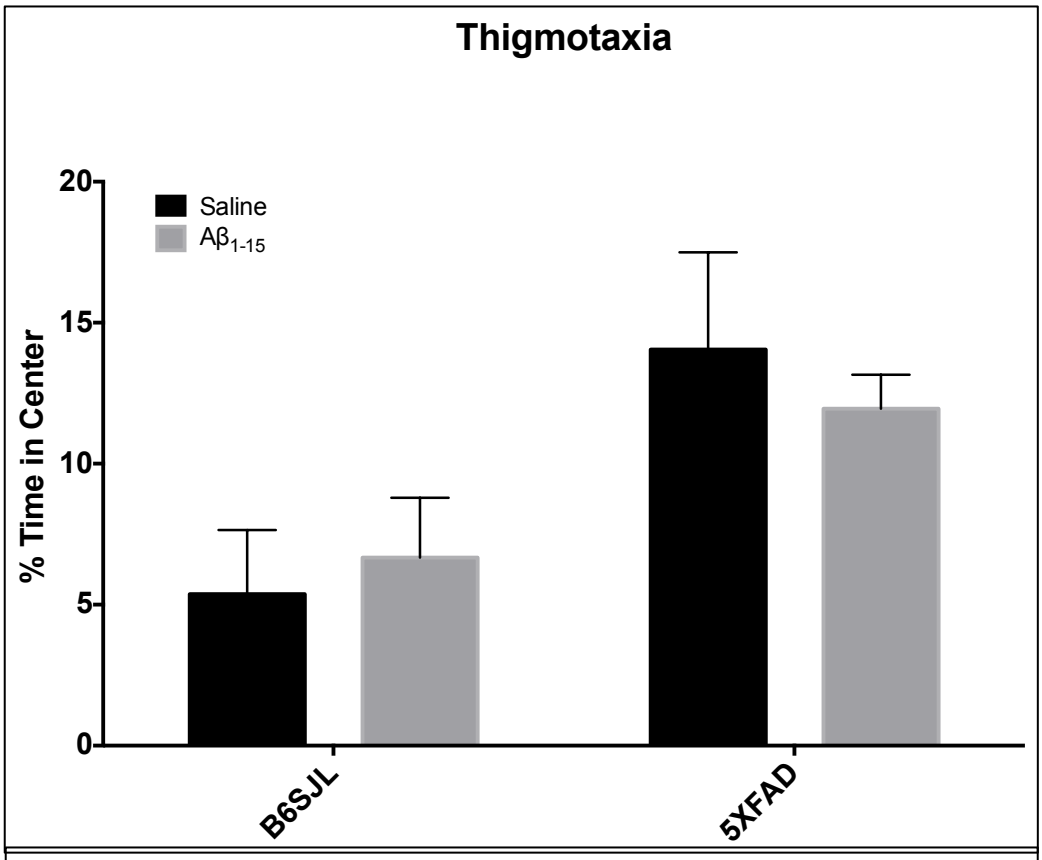

Figure 30. **5XFAD mice exhibit hyperexploratory behavior with more time spent in the center of an open field**. Thigmotaxia in open field for B6SJL vs. 5XFAD mice was measured via TSE videotracking software Data are means ± SEM, n=3/group for B6SJL, n=7/group for 5XFAD.

Total distance traveled was also collected on each day to rule out any motor deficits that could affect the NOR (or CFC) test results. On the first day of NOR there were significant differences in the total distance traveled between the B6.SJL group injected with $A\beta_{1-15}$ versus both the 5XFAD group injected with saline (Fig. 31, $p<0.05$) and the 5XFAD group injected with $A\beta_{1-15}$ (Fig. 31, $p<0.01$). On the second day of NOR there were no significant differences in total distance traveled between groups but there was a significant increase in total distance traveled, likely due to the objects being explored.

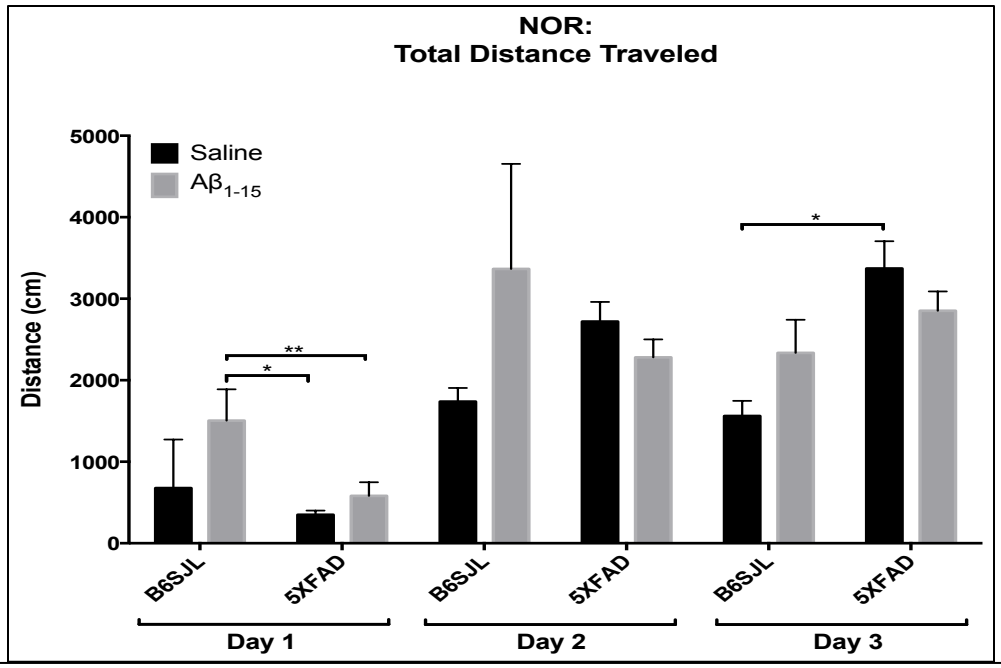

Figure 31. **Total distance traveled on days 1, 2 and 3 of NOR showed significant differences between WT and 5XFAD mice**. 500nM $A\beta_{1-15}$ or sterile saline were bilaterally injected into the dorsal hippocampi 20mins before day 2 training. Total distance travelled in an open field was measured via TSE videotracking software *$p<0.05$ showing a significant difference between B6SJL mice injected with $A\beta_{1-15}$ compared to 5XFAD mice injected with saline; **$p<0.005$ 5XFAD injected with $A\beta_{1-15}$ on day 1; *$p<0.05$ significant differences between B6SJL mice treated with saline compared to 5XFAD mice treated with saline on Day 3. Data are means ± SEM, n=3/group for B6SJL, n=7/group for 5XFAD.

Day 3 of NOR showed similar distances traveled as day 2 with a significant difference of total distance traveled between B6.SJL and 5XFAD mice treated with the N-terminal fragment (Fig. 31).

Novel object recognition test results showed all groups had a preference for the novel object except for the B6.SJL saline group, with no significant differences between the groups with the preference for the novel object (Fig. 31).

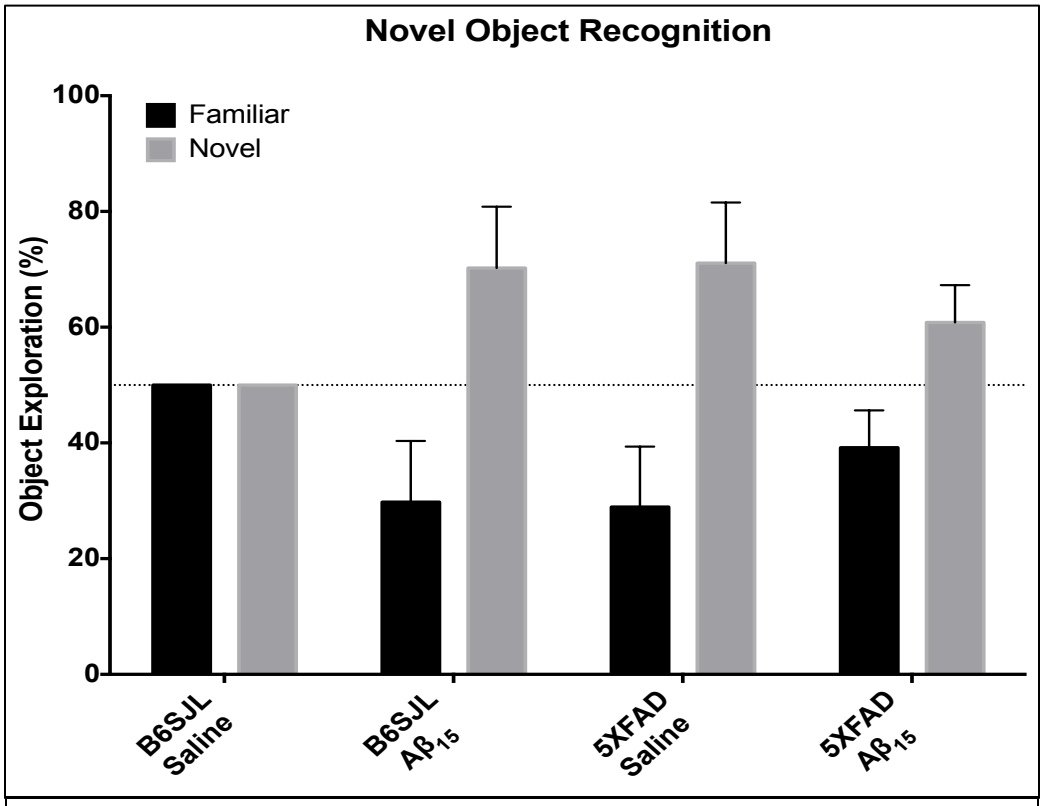

Figure 32. **No differences exhibited in novel object preference in B6.SJL and 5XFAD mice injected with A$\beta_{1\text{-}15}$ or saline**. 500nM A$\beta_{1\text{-}15}$ or sterile saline were bilaterally injected into the dorsal hippocampi 20mins before day 2 training. Time spent exploring each object was measured via TSE videotracking software and data are presented as percent exploration with exploration over 50% indicating a preference for that object. Data are means of percent of time spent with each object Data are means ± SEM, n=3/group for B6SJL, n=7/group for 5XFAD.

Elevated Plus Maze:

The elevated plus maze is a well-established test of anxiety. We investigated if the injection of 0.40μL of 500nM A $\beta_{1\text{-}15}$ would have an effect on the 5XFAD mice which tend to be hyper-exploratory compared to their wild-type counterparts.[83] We first looked at total distance traveled to rule out any motor deficits and found no significant differences between groups (Fig. 33).

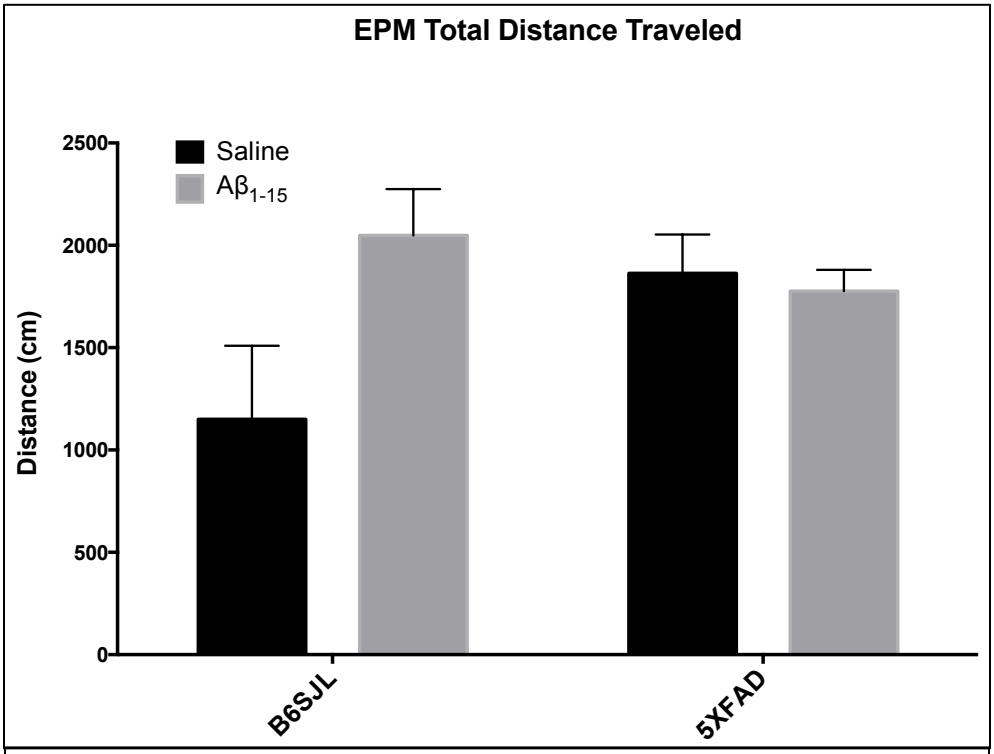

Figure 33. **No significant differences in distance travelled between groups in elevated plus maze**. Distance traveled in WT and 5XFAD mice injected with 500nM A $\beta_{1\text{-}15}$ or saline into the dorsal hippocampus 24h before EPM. Total distance traveled was collected using TSE videotracking software. Data are means ±SEM, n=3/group for B6SJL, n=7/group for 5XFAD.

After $A\beta_{1\text{-}15}$ injections, 5XFAD mice spent significantly more time exploring open arms, signifying a decrease in anxiety (Fig. 34, $p<0.05$). B6.SJL mice showed a non-significant but similar trend.

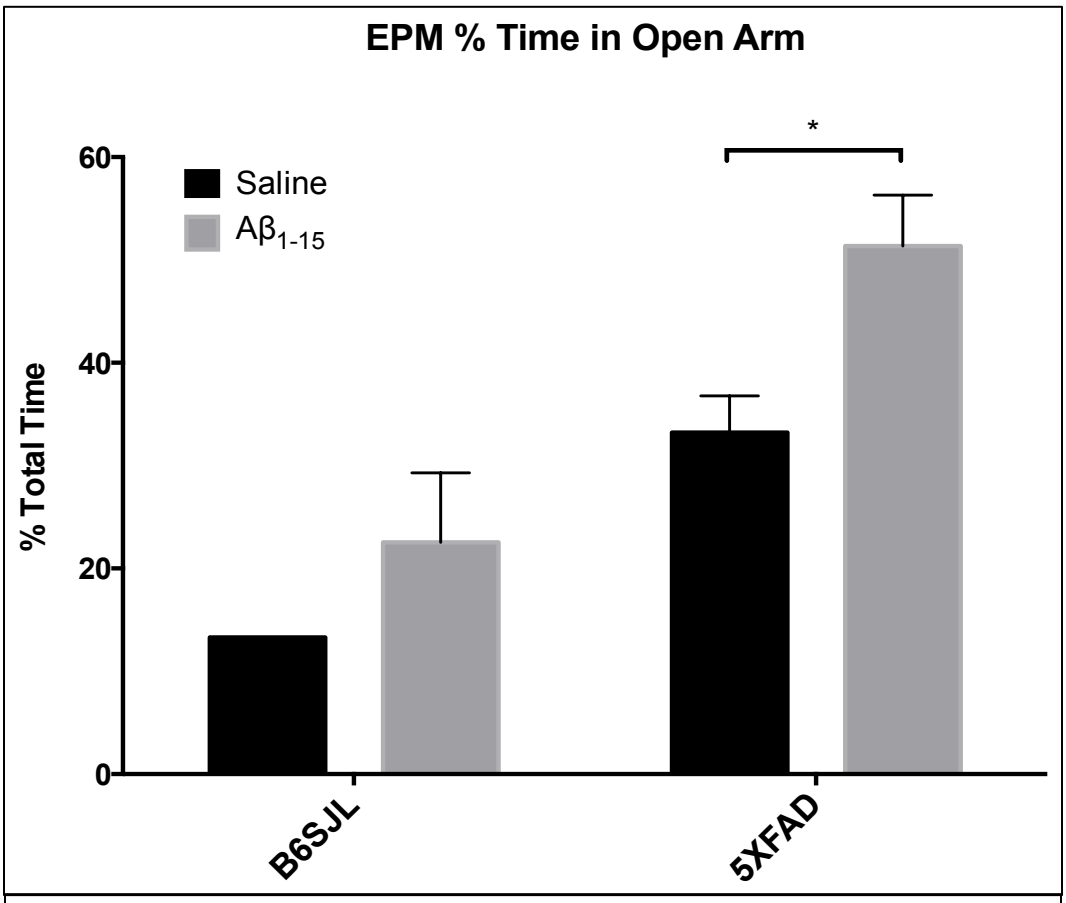

Figure 34. **5XFAD mice injected with A$\beta_{1\text{-}15}$ exhibit lower anxiety compared to saline injected 5XFAD mice.** Percent of time spent in open arms in WT and 5XFAD mice injected with 500nM A$\beta_{1\text{-}15}$ or saline into the dorsal hippocampus 24h before EPM test was measured by TSE videotracking software and percent of time spent in open arms is plotted. (*$p<0.05$) Data are means ±SEM, n=2-3/group for B6SJL, n=7/group for 5XFAD.

Intracellular Signaling – Immunoblot analysis:

Lastly, we preliminarily explored possible molecular changes occurring with $A\beta_{1\text{-}15}$ injection by extracting compartment proteins from hippocampi from B6.SJL and 5XFAD mice injected with $A\beta_{1\text{-}15}$, or saline. We probed for changes in pJNK and pERK. We also explored changes in receptor regulation by looking at NMDAR subunit NR1 and AMPAR subunit, GluR2 in the membrane fraction, both of which are essential for LTP. Lastly, we investigated pCREB signaling in the nuclear fraction. CREB activation is essential for memory processing and is perturbed in AD patients.[84–86]

We observed a clear downregulation of NR1 subunits in the membrane fraction of 5XFAD animals compared to B6.SJL mice. The B6.SJL mice injected with $A\beta_{1\text{-}15}$ exhibited a slight decrease in NR1, while the 5XFAD mice injected with $A\beta_{1\text{-}15}$ displayed a slight increase in NR1 (Fig. 35).

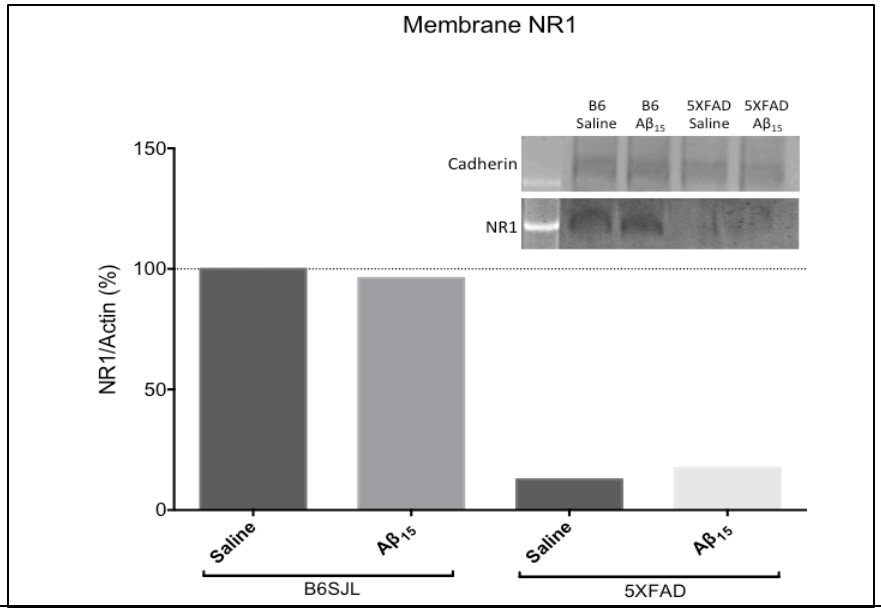

Figure 35. **Membrane NR1 is lower in 5XFAD mice compared to WT counterparts.** Hippocampi collected 24h after microinjections of 500nM $A\beta_{1\text{-}15}$ or saline in B6.SJL and 5XFAD mice. Compartment protein extraction was achieved using a compartmental protein extraction kit. Mean signal normalized to Cadherin loading control compared to saline injected B6SJL mice. Lane 1 upper and lower panel: 100kDa. n=1/group

Similarly, GluR2 levels are lower in the saline-injected 5XFAD mice compared to the saline-injected wild-type B6.SJL mice. Injection of $A\beta_{1-15}$ induced an upregulation of AMPA receptor subunit GluR2 in both the B6.SJL mice as well as the 5XFAD mice (Fig. 36).

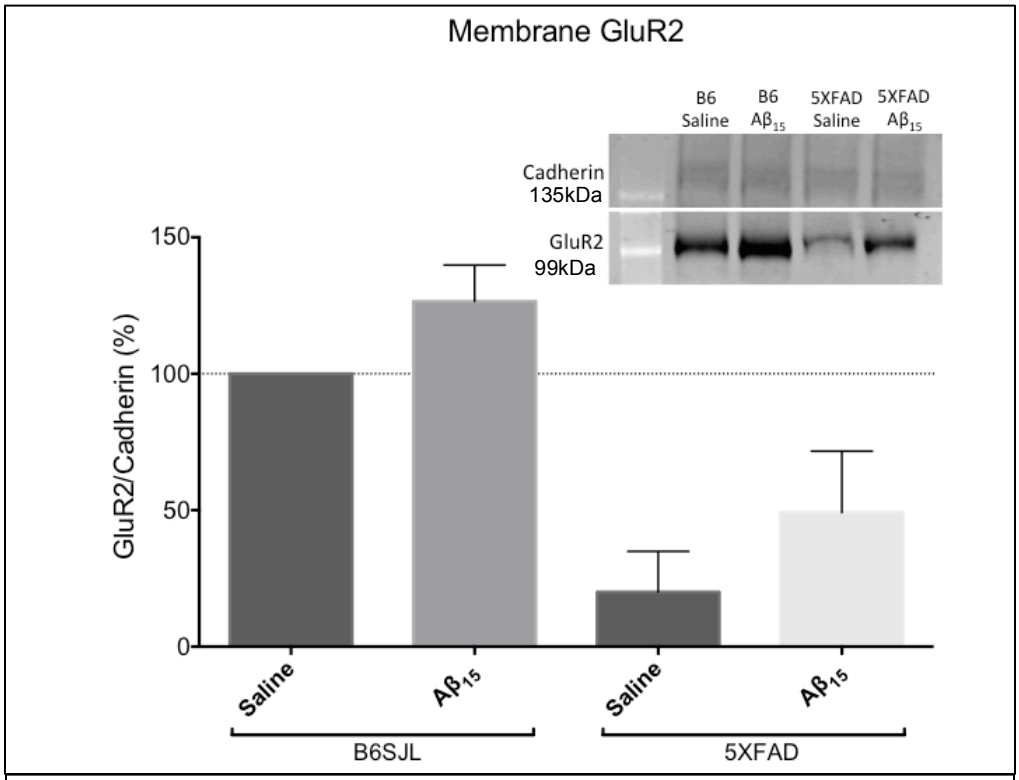

Figure 36. **GluR2 levels are lower in 5XFAD mice compared to WTs with A β $_{1-15}$ injection increasing mGluR2 levels in bot 5XFAD and WT mice.** mGluR2 mean signal normalized to cadherin loading control compared to saline injected B6SJL mice. Hippocampi collected 24h after microinjections of 500nM $A\beta_{1-15}$ or saline in B6.SJL and 5XFAD mice. Compartment protein extraction was achieved using a compartmental protein extraction kit. Lane 1 upper and lower panel: 100kDa. n=3/group.

Total CREB levels were found to be substantially lower in 5XFAD mouse hippocampi in comparison to the B6.SJL mouse hippocampi, consistent with previous findings.[84] Injection of the N-terminal $A\beta_{1-15}$ fragment led to upregulation of phosphorylated CREB in both wild-type and 5XFAD mice, with a more pronounced (2-fold) upregulation in 5XFAD mice (Fig. 37). There was only a modest, insignificant increase in total CREB in the 5XFAD hippocampi with injection of $A\beta_{1-15}$.

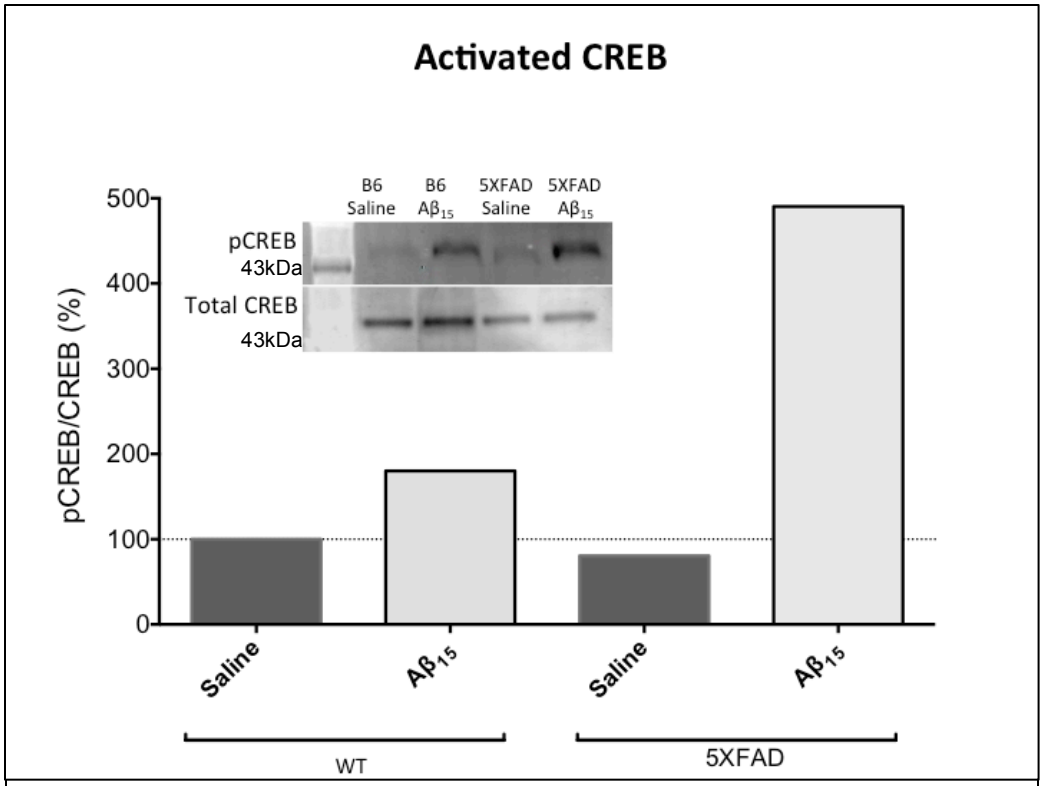

Figure 37. **pCREB levels are lower in 5XFAD mice compared to WTs with $A\beta_{1-15}$ injection increasing pCREB in both 5XFAD and WT mice.** pCREB mean signal normalized to total CREB previously normalized to actin loading control (from cytoplasmic fraction of the same samples). Hippocampi collected 24h after microinjections of 500nM $A\beta_{1-15}$ or saline in B6.SJL and 5XFAD mice. Compartment protein extraction was achieved using a compartmental protein extraction kit. Lane 1 upper and lower panel: 37kDa. n=1/group.

Discussion

Current studies examining cognition in AD mouse models have used everything from metal chelating agents to HDAC6 inhibitors, β-secretase inhibitors, and even anti-epileptic drugs to rescue memory.[77,87,88] Many of these agents have deleterious effects due to the presence of multiple substrates limiting the specificity of action or have very limited beneficial effects. Even current treatments like NMDA antagonists and cholinesterase inhibitors are proving to only be useful in the early stages of AD, and only for limited symptomatic treatment among a limited population of patients.[75,89]

In this study we show that $A\beta_{1-15}$ is able to rescue (restore) memory in CFC up to twelve days post training in 5XFAD mice, and up to 7.5 months of age. This is very promising considering this model has accelerated Aβ production with intraneuronal Aβ deposits observed at 2 months of age. Thus, this treatment shows potential for positive effects late in the pathology of AD.[90] The mechanism by which this occurs is still unknown. We were also able to demonstrate a decrease in anxiety via EPM in 5XFAD mice injected with $A\beta_{1-15}$ compared to saline injected mice with a similar trend in B6.SJL mice suggesting an anxiolytic effect of $A\beta_{1-15}$. Furthermore, all animals showed a preference for the novel object in NOR, suggesting that recognition memory was not affected in 5XFAD mice, though the exploratory behavior was.

We investigated changes in intracellular signaling induced by bilateral $A\beta_{15}$ injections into the hippocampi of the mice as first, preliminary step toward uncovering the means by which the N-terminal Aβ peptide rescues 5XFAD memory deficits. We focused on mGluR and NR1 levels, subunits of AMPA receptors and NMDA receptors,

respectively, and pCREB. As major players in long-term potentiation and memory, changes in these proteins would most reflect changes in LTP and behavior.

We observed a slight upregulation of NR1 in $A\beta_{15}$ injected 5XFAD mice. In contrast, mice injected with $A\beta_{15}$ had a very pronounced upregulation of both mGluR2 and activated CREB compared to their saline-injected counterparts. This gives us a clue as to what could possibly be occurring with $A\beta_{15}$ injection or perfusion. We know that activated CREB (pCREB) is able to bind BDNF promoters and increase AMPA receptor activity (ref). AMPA receptor insertion is imperative for LTP and this upregulation can partially explain the memory enhancement observed in the $A\beta_{15}$ injected 5XFAD mice, which otherwise show substantial memory deficits. Altered regulation and expression of CREB has been identified as a central signaling deficit in Alzheimer's disease[85,86], as CREB activity is crucial to memory[91,92] That pCREB levels were corrected with $A\beta_{1\text{-}15}$ injection in the 5XFAD hippocampus while also significantly upregulated in the wild-type hippocampus strongly supports the direct effect of the N-terminal $A\beta_{1\text{-}15}$ fragment peptide on the primary signaling pathways for memory processing.

Previous studies have also indicated that chronic $A\beta_{42}$ application to hippocampal slice cultures activates JNK/MAPK and inhibits ERK/MAPK pathways, suggesting a way that $A\beta_{42}$ impairs memory, due to the fact that ERK1/2 are important signaling molecules in learning and memory.[35] While our data were inconclusive for JNK and ERK (data not shown), these would be important to further explore to determine if $A\beta_{1\text{-}15}$ is able to prevent the effects of the toxic $A\beta_{42}$ on JNK and ERK activation. With such a robust

phenotypic enhancement, further studies investigating how the N-terminal fragment enhances memory and prevents actions of the full-length toxic peptide are essential to further elucidating its mechanism of action.

CHAPTER 5

Conclusions

Concluding Remarks

Previous work in the Nichols lab established a toxicity timeline for $A\beta_{1-42}$ in the model neuronal system we utilize, and confirmed its activation of MAPK-linked toxicity pathways.[57] In conjunction with that, we have also previously shown that the $A\beta_{1-15}$ fragment, which is endogenously present in the CSF of healthy adults, indicating its presence in brain, is highly potent and more effective than $A\beta_{1-42}$ in its agonist-like action via nAChRs.[2] Due to its short, hydrophilic, nontoxic nature, we hypothesized that $A\beta_{1-15}$ could have a neuroprotective role against the toxic full length $A\beta_{1-42}$.

This study confirmed that $A\beta_{1-15}$ is neuroprotective in the differentiated NG108-15 neuronal cell model by assessing non-specific ROS production as well as nuclear fragmentation that has been previously reported following treatment of cells with toxic, full-length Aβ. $A\beta_{1-15}$ treatment with various dosages and timing showed the N-terminal fragment protected against the well-established $A\beta_{42}$ toxicity (Figs. 11 & 12). We also examined cell survival over one week treatment to examine whether the neuroprotection in the NG108-15 cells was limited to the ROS study timeline of three days. We observed that the neuroprotection by $A\beta_{1-15}$ persisted throughout the seven-day time course, showing a lasting neuroprotective effect of $A\beta_{1-15}$ (Fig. 13).

To further investigate this neuroprotection, we then examined this effect in the more physiologically relevant primary hippocampal neuron culture. We investigated toxicity and neuroprotection at different time points (5 and 10 days), examining the ability of $A\beta_{1-15}$ to protect against $A\beta_{42}$ toxicity. As hypothesized, it was necessary to use higher concentrations (1μM) of $A\beta_{42}$ to kill the primary cultures, and $A\beta_{1-15}$, at the

same concentration, or even at 100nM, was able to protect these cultures from $A\beta_{42}$ toxicity (Fig. 15 & 16). Similar to cell-survival in the NG108-15 cell line, we examined $A\beta_{1-15}$ neuroprotection in the primary neurons over 10 days and observed similar ability of the N-terminal fragment to significantly protect against $A\beta_{42}$ toxicity (Fig.18). The results from these model neuronal systems indicate several possible actions by the N-terminal fragment in neuroprotection, including competitive block of $A\beta_{42}$, anti-apoptotic signaling, and anti-oxidative pathway activation. The extent these possibilities underlie the neuroprotective action of $A\beta_{1-15}$ remains to be determined.

Furthermore, $A\beta_{42}$ has been shown to perturb synaptic function and prevent long-term potentiation, so we studied the effects of $A\beta_{1-15}$ on LTP and examined whether it could prevent the LTP block by $A\beta_{42}$.[7,64] These electrophysiological studies showed that $A\beta_{1-15}$ was able to enhance LTP in wild-type acute hippocampal slices at very low concentrations as well as prevent the block of LTP by $A\beta_{42}$ (Fig. 20). In fact, perfusion of slices with $A\beta_{1-15}$ for 20 minutes before the perfusion of $A\beta_{42}$ completely ameliorates any LTP deficits caused by $A\beta_{42}$ and allows for LTP at normal control levels. The most promising of the LTP experiments examined LTP rescue in APPswe mice. APPswe mice have a familial Swedish mutation that causes accelerated Aβ production and have been reported to have LTP deficits. The perfusion of brain slices with $A\beta_{1-15}$ was able to rescue LTP to control levels (Fig. 21). Toxic $A\beta_{42}$ perturbs LTP by reducing NMDARs, and prompting NMDAR-mediated calcium influx inducing excitotoxicity, and activating stress pathways that result in synaptic dysfunction. Our electrophysiology results suggest that $A\beta_{1-15}$ might prevent LTP inhibition by $A\beta_{42}$ due to competitive block, or

upregulation of AMPARs. Further studies are required to determine the specific mechanisms involved in $A\beta_{1-15}$ LTP rescue.

To complement the electrophysiology experiments, we explored different behavior paradigms using the 5XFAD mouse model: a mouse model for AD with 3 different familial APP mutations and two presenilin mutations, which exhibits accelerated Aβ production, leading to memory deficits within 6 months of age. We showed that $A\beta_{1-15}$ was able to rescue fear memory in contextual fear conditioning as well as reduce anxiety in the elevated plus maze further cementing $A\beta_{1-15}$'s ability to rescue memory (Fig. 27, 28 & 34). In parallel with behavior, we explored changes in membrane NR1 and GluR2 levels as well as activated CREB and observed a very large upregulation of GluR2 and phosphorylated CREB giving us insight into the actions of $A\beta_{1-15}$ (Fig. 35-37). Of particular note, CREB has been linked to memory in the brain, and the $A\beta_{1-15}$-mediated reversal of its downregulation by $A\beta_{42}$ may be part of the N-terminal fragments primary action to rescue the memory deficits in the 5XFAD mice.

Taken together, the current study highlights the neuroprotective effects of $A\beta_{1-15}$ against oxidative stress and neurodegeneration caused by $A\beta_{42}$, its ability to prevent synaptic dysfunction, and its ability to rescue memory deficits in an AD mouse model. These data support the hypothesis that $A\beta_{1-15}$ is neuroprotective against $A\beta_{42}$ toxicity mediated by any and all of its target receptors. The capability to show these effects by an endogenous peptide in physiologically relevant models is confirmation that this peptide could lead to a new avenue for AD therapies.

Future Directions

This study has established that the N-terminal fragment, $A\beta_{1-15}$ has a neuromodulatory function, is neuroprotective against $A\beta_{42}$ toxicity in neuroblastoma and hippocampal primary neuron cultures, is able to enhance LTP, prevent $A\beta_{42}$ LTP block, rescue LTP in APPswe mice, and is able rescue contextual fear memory in 5XFAD mice. While this peptide shows high potential to be a candidate for AD therapy, there are many avenues that should be explored further. These include, but are not limited to:

1. Determine the crystal structure for both $A\beta_{1-15}$ and $A\beta_{10-15}$ in complex with nAChRs to determine their precise binding into the ligand-activation pocket. This will provide important information for future derivation of the N-terminal Aβ fragment peptides, particular for development of peptidomimetics.
2. Binding by $A\beta_{1-15}$ should be explored in depth. This could be achieved via membrane binding studies to determine steady-state kinetic parameters (IC_{50}) and competitive binding between $A\beta_{1-15}$ and $A\beta_{42}$. This will also provide essential information for development of peptide derivatives.
3. Examine how $A\beta_{1-15}$ is taken up by the neurons and the amount of time it takes for it to be degraded by tagging $A\beta_{1-15}$ and looking at the amounts in serum and brain tissue in a time-dependent manner after injection.
4. Investigate changes in synapse morphology and/or synapse density with $A\beta_{1-15}$ vs. saline (acute vs. chronic treatment) in 5XFAD mice. This can be achieved using Golgi staining or electron microscopy.

5. Larger scale electrophysiology and behavior experiments with more elaborate controls should also be performed. We can explore whether N-terminal Aβ peptides can promote sub-threshold potentiation, which can push short-term potentiation to long-term potentiation. In addition, the effect of $A\beta_{1-15}$ on LTD should also be explored, as the LTD mechanism is as important as LTP for learning and memory.
6. The N-terminal core fragment $A\beta_{10-15}$ should be more intensively studied. As the core of the N-terminal fragment is responsible for its activity, it has high potential to be developed as a peptidomimetic, optimized to cross the blood-brain barrier. (The original peptides would be predicted to poorly cross the blood-barrier.) If an identified peptidomimetic is confirmed to have similar effects as that seen with the $A\beta_{1-15}$ and $A\beta_{10-15}$ in both LTP and behavior, pharmacokinetic studies could then be performed, examining oral or intranasal bioavailability for potential therapeutic application.

REFERENCES

1. Holtzman, D. M., Morris, J. C. & Goate, A. M. Alzheimer's Disease: The Challenge of the Second Century. *Sci. Transl. Med.* **3,** 77sr1–77sr1 (2011).
2. Lawrence, J. L. M., Tong, M., Alfulaij, N., Sherrin, T., Contarino, M., White, M. M., Bellinger, F. P., Todorovic, C. & Nichols, R. A. Regulation of Presynaptic Ca2+, Synaptic Plasticity and Contextual Fear Conditioning by a N-terminal β-Amyloid Fragment. *J. Neurosci.* **34,** 14210–14218 (2014).
3. Enhancing memory with IGF-II. *Enhancing Mem. IGF-II* at <http://igf-ii-memory.weebly.com/>
4. Zhou, M., Greenhill, S., Huang, S., Silva, T. K., Sano, Y., Wu, S., Cai, Y., Nagaoka, Y., Sehgal, M., Cai, D. J., Lee, Y.-S., Fox, K. & Silva, A. J. CCR5 is a suppressor for cortical plasticity and hippocampal learning and memory. *eLife* **5,** (2016).
5. Latest Facts & Figures Report | Alzheimer's Association. at <http://www.alz.org/alzheimers_disease_facts_and_figures.asp#prevalence>
6. Stelzmann, R. A., Norman Schnitzlein, H. & Reed Murtagh, F. An English translation of Alzheimer's 1907 paper,'Über eine eigenartige Erkankung der Hirnrinde'. *Clin. Anat.* **8,** 429–431 (1995).
7. Puzzo, D., Privitera, L. & Palmeri, A. Hormetic effect of amyloid-beta peptide in synaptic plasticity and memory. *Neurobiol. Aging* **33,** 1484.e15–1484.e24 (2012).
8. Puzzo, D., Privitera, L., Fa', M., Staniszewski, A., Hashimoto, G., Aziz, F., Sakurai, M., Ribe, E. M., Troy, C. M., Mercken, M., Jung, S. S., Palmeri, A. & Arancio, O.

Endogenous amyloid-β is necessary for hippocampal synaptic plasticity and memory. *Ann. Neurol.* **69,** 819–830 (2011).

9. Puzzo, D., Privitera, L., Leznik, E., Fà, M., Staniszewski, A., Palmeri, A. & Arancio, O. Picomolar Amyloid-β Positively Modulates Synaptic Plasticity and Memory in Hippocampus. *J. Neurosci.* **28,** 14537–14545 (2008).
10. Portelius, E., Price, E., Brinkmalm, G., Stiteler, M., Olsson, M., Persson, R., Westman-Brinkmalm, A., Zetterberg, H., Simon, A. J. & Blennow, K. A novel pathway for amyloid precursor protein processing. *Neurobiol. Aging* **32,** 1090–1098 (2011).
11. Palop, J. J. & Mucke, L. Amyloid-β-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nat. Neurosci.* **13,** 812–818 (2010).
12. Coburger, I., Hoefgen, S. & Than, M. E. The structural biology of the amyloid precursor protein APP – a complex puzzle reveals its multi-domain architecture. *Biol. Chem.* **395,** 485–498 (2014).
13. Turner, P. R., O'Connor, K., Tate, W. P. & Abraham, W. C. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Prog. Neurobiol.* **70,** 1–32 (2003).
14. Zhang, Y., Thompson, R., Zhang, H. & Xu, H. APP processing in Alzheimer's disease. *Mol. Brain* **4,** 3 (2011).
15. Poulsen, E. T., Iannuzzi, F., Rasmussen, H. F., Maier, T. J., Enghild, J. J., Jørgensen, A. L. & Matrone, C. An Aberrant Phosphorylation of Amyloid Precursor Protein Tyrosine Regulates Its Trafficking and the Binding to the Clathrin Endocytic

Complex in Neural Stem Cells of Alzheimer's Disease Patients. *Front. Mol. Neurosci.* **10,** 59 (2017).

16. De Strooper, B., Vassar, R. & Golde, T. The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat. Rev. Neurol.* **6,** 99–107 (2010).
17. Octave, J.-N., Pierrot, N., Ferao Santos, S., Nalivaeva, N. N. & Turner, A. J. From synaptic spines to nuclear signaling: nuclear and synaptic actions of the amyloid precursor protein. *J. Neurochem.* **126,** 183–190 (2013).
18. De Strooper, B., Vassar, R. & Golde, T. The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat. Rev. Neurol.* **6,** 99–107 (2010).
19. Strooper, B. D. & Annaert, W. Proteolytic processing and cell biological functions of the amyloid precursor protein. *J. Cell Sci.* **113,** 1857–1870 (2000).
20. Cole, S. L. & Vassar, R. The Alzheimer's disease β-secretase enzyme, BACE1. *Mol. Neurodegener.* **2,** 22 (2007).
21. LaFerla, F. M., Green, K. N. & Oddo, S. Intracellular amyloid-β in Alzheimer's disease. *Nat. Rev. Neurosci.* **8,** 499–509 (2007).
22. Webb, R. L. & Murphy, M. P. β-Secretases, Alzheimer's Disease, and Down Syndrome. *Curr. Gerontol. Geriatr. Res.* **2012,** 1–8 (2012).
23. Mokhtar, S. H., Bakhuraysah, M. M., Cram, D. S. & Petratos, S. The Beta-Amyloid Protein of Alzheimer’s Disease: Communication Breakdown by Modifying the Neuronal Cytoskeleton. *Int. J. Alzheimer's Dis.* **2013,** (2013).
24. Salloway, S., Mintzer, J., Weiner, M. F. & Cummings, J. L. Disease-modifying therapies in Alzheimer's disease. *Alzheimers Dement.* **4,** 65–79 (2008).

25. Mawuenyega, K. G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J. C., Yarasheski, K. E. & Bateman, R. J. Decreased Clearance of CNS β-Amyloid in Alzheimer's Disease. *Science* **330,** 1774–1774 (2010).
26. Morley, J. E., Farr, S. A., Banks, W. A., Johnson, S. N., Yamada, K. A. & Xu, L. A Physiological Role for Amyloid-β Protein: Enhancement of Learning and Memory. *J. Alzheimers Dis.* **19,** 441–449 (2010).
27. Puzzo, D. & Arancio, O. Amyloid-β Peptide: Dr. Jekyll or Mr. Hyde? *J. Alzheimers Dis.* **33,** S111–S120 (2013).
28. Nutu, M., Bourgeois, P., Zetterberg, H., Portelius, E., Andreasson, U., Parent, S., Lipari, F., Hall, S., Constantinescu, R., Hansson, O. & Blennow, K. Aβ1-15/16 as a Potential Diagnostic Marker in Neurodegenerative Diseases. *NeuroMolecular Med.* **15,** 169–179 (2013).
29. Portelius, E., Zetterberg, H., Andreasson, U., Brinkmalm, G., Andreasen, N., Wallin, A., Westman-Brinkmalm, A. & Blennow, K. An Alzheimer's disease-specific β-amyloid fragment signature in cerebrospinal fluid. *Neurosci. Lett.* **409,** 215–219 (2006).
30. Portelius, E., Zetterberg, H., Dean, R. A., Marcil, A., Bourgeois, P., Nutu, M., Andreasson, U., Siemers, E., Mawuenyega, K. G., Sigurdson, W. C., May, P. C., Paul, S. M., Holtzman, D. M., Blennow, K. & Bateman, R. J. Amyloid-β1-15/16 as a Marker for γ-Secretase Inhibition in Alzheimer's Disease. *J. Alzheimers Dis.* **31,** 335–341 (2012).

31. Portelius, E., Van Broeck, B., Andreasson, U., Gustavsson, M. K., Mercken, M., Zetterberg, H., Borghys, H. & Blennow, K. Acute Effect on the Aβ Isoform Pattern in CSF in Response to γ-Secretase Modulator and Inhibitor Treatment in Dogs. *J. Alzheimers Dis.* **21,** 1005–1012 (2010).
32. Cook, J. J., Wildsmith, K. R., Gilberto, D. B., Holahan, M. A., Kinney, G. G., Mathers, P. D., Michener, M. S., Price, E. A., Shearman, M. S., Simon, A. J., Wang, J. X., Wu, G., Yarasheski, K. E. & Bateman, R. J. Acute γ-Secretase Inhibition of Nonhuman Primate CNS Shifts Amyloid Precursor Protein (APP) Metabolism from Amyloid-β Production to Alternative APP Fragments without Amyloid-β Rebound. *J. Neurosci.* **30,** 6743–6750 (2010).
33. Pettit, D. L., Shao, Z. & Yakel, J. L. β-Amyloid1–42 Peptide Directly Modulates Nicotinic Receptors in the Rat Hippocampal Slice. *J. Neurosci.* **21,** RC120–RC120 (2001).
34. Okada, H., Ouchi, Y., Ogawa, M., Futatsubashi, M., Saito, Y., Yoshikawa, E., Terada, T., Oboshi, Y., Tsukada, H., Ueki, T., Watanabe, M., Yamashita, T. & Magata, Y. Alterations in α4β2 nicotinic receptors in cognitive decline in Alzheimer's aetiopathology. *Brain* **136,** 3004–3017 (2013).
35. Buckingham, S. D., Jones, A. K., Brown, L. A. & Sattelle, D. B. Nicotinic Acetylcholine Receptor Signalling: Roles in Alzheimer's Disease and Amyloid Neuroprotection. *Pharmacol. Rev.* **61,** 39–61 (2009).

36. Tsetlin, V. & Kasheverov, I. in *Neurochemistry* (ed. Heinbockel, T.) (InTech, 2014). at <http://www.intechopen.com/books/neurochemistry/peptide-and-protein-neurotoxin-toolbox-in-research-on-nicotinic-acetylcholine-receptors>
37. Parri, H. R., Hernandez, C. M. & Dineley, K. T. Research update: Alpha7 nicotinic acetylcholine receptor mechanisms in Alzheimer's disease. *Biochem. Pharmacol.* **82,** 931–942 (2011).
38. Chin, J. H., Ma, L., MacTavish, D. & Jhamandas, J. H. Amyloid β Protein Modulates Glutamate-Mediated Neurotransmission in the Rat Basal Forebrain: Involvement of Presynaptic Neuronal Nicotinic Acetylcholine and Metabotropic Glutamate Receptors. *J. Neurosci.* **27,** 9262–9269 (2007).
39. Arora, K., Alfulaij, N., Higa, J. K., Panee, J. & Nichols, R. A. Impact of Sustained Exposure to β-Amyloid on Calcium Homeostasis and Neuronal Integrity in Model Nerve Cell System Expressing α4β2 Nicotinic Acetylcholine Receptors. *J. Biol. Chem.* **288,** 11175–11190 (2013).
40. Wang, H.-Y., Lee, D. H. S., D'Andrea, M. R., Peterson, P. A., Shank, R. P. & Reitz, A. B. β-Amyloid1–42 Binds to α7 Nicotinic Acetylcholine Receptor with High Affinity IMPLICATIONS FOR ALZHEIMER'S DISEASE PATHOLOGY. *J. Biol. Chem.* **275,** 5626–5632 (2000).
41. Xia, M., Cheng, X., Yi, R., Gao, D. & Xiong, J. The Binding Receptors of Aβ: an Alternative Therapeutic Target for Alzheimer's Disease. *Mol. Neurobiol.* **53,** 455–471 (2016).

42. Ostapchenko, V. G., Beraldo, F. H., Mohammad, A. H., Xie, Y.-F., Hirata, P. H. F., Magalhaes, A. C., Lamour, G., Li, H., Maciejewski, A., Belrose, J. C., Teixeira, B. L., Fahnestock, M., Ferreira, S. T., Cashman, N. R., Hajj, G. N. M., Jackson, M. F., Choy, W.-Y., MacDonald, J. F., Martins, V. R., Prado, V. F. & Prado, M. A. M. The Prion Protein Ligand, Stress-Inducible Phosphoprotein 1, Regulates Amyloid-β Oligomer Toxicity. *J. Neurosci.* **33,** 16552–16564 (2013).
43. Um, J. W., Nygaard, H. B., Heiss, J. K., Kostylev, M. A., Stagi, M., Vortmeyer, A., Wisniewski, T., Gunther, E. C. & Strittmatter, S. M. Alzheimer amyloid-β oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat. Neurosci.* **15,** 1227–1235 (2012).
44. Nelson, P., Christian, C. & Nirenberg, M. Synapse formation between clonal neuroblastoma X glioma hybrid cells and striated muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **73,** 123 (1976).
45. McGee, R., Simpson, P., Christian, C., Mata, M., Nelson, P. & Nirenberg, M. Regulation of acetylcholine release from neuroblastoma x glioma hybrid cells. *Proc. Natl. Acad. Sci. U. S. A.* **75,** 1314 (1978).
46. Rondé, P., Dougherty, J. J. & Nichols, R. A. Functional IP3- and ryanodine-sensitive calcium stores in presynaptic varicosities of NG108-15 (rodent neuroblastoma x glioma hybrid) cells. *J. Physiol.* **529 Pt 2,** 307–319 (2000).
47. Gordon, J., Amini, S. & White, M. K. General overview of neuronal cell culture. *Methods Mol. Biol. Clifton NJ* **1078,** 1–8 (2013).

48. Dotti, C. G., Sullivan, C. A. & Banker, G. A. The establishment of polarity by hippocampal neurons in culture. *J. Neurosci. Off. J. Soc. Neurosci.* **8,** 1454–1468 (1988).
49. Matteoli, M., Verderio, C., Krawzeski, K., Mundigl, O., Coco, S., Fumagalli, G. & De Camilli, P. Mechanisms of synaptogenesis in hippocampal neurons in primary culture. *J. Physiol.-Paris* **89,** 51–55 (1995).
50. Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A. & Van Eldik, L. J. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front. Genet.* **5,** 88 (2014).
51. Head, E. A canine model of human aging and Alzheimer's disease. *Biochim. Biophys. Acta* **1832,** 1384–1389 (2013).
52. Bateman, R. J., Munsell, L. Y., Morris, J. C., Swarm, R., Yarasheski, K. E. & Holtzman, D. M. Human amyloid-β synthesis and clearance rates as measured in cerebrospinal fluid in vivo. *Nat. Med.* **12,** 856–861 (2006).
53. Sherrin, T., Blank, T., Hippel, C., Rayner, M., Davis, R. J. & Todorovic, C. Hippocampal c-Jun-N-Terminal Kinases Serve as Negative Regulators of Associative Learning. *J. Neurosci.* **30,** 13348–13361 (2010).
54. Jia, Q., Deng, Y. & Qing, H. Potential Therapeutic Strategies for Alzheimer’s Disease Targeting or Beyond *β*-Amyloid: Insights from Clinical Trials. *BioMed Res. Int.* **2014,** e837157 (2014).

55. Puzzo, D., Lee, L., Palmeri, A., Calabrese, G. & Arancio, O. Behavioral assays with mouse models of Alzheimer's disease: Practical considerations and guidelines. *Biochem. Pharmacol.* **88,** 450–467 (2014).
56. Mayeux, R. & Stern, Y. Epidemiology of Alzheimer Disease. *Cold Spring Harb. Perspect. Med.* **2,** a006239–a006239 (2012).
57. Arora, K., Cheng, J. & Nichols, R. A. Nicotinic Acetylcholine Receptors Sensitize a MAPK-linked Toxicity Pathway on Prolonged Exposure to β-Amyloid. *J. Biol. Chem.* **290,** 21409–21420 (2015).
58. Cheng, Q. & Yakel, J. L. Activation of α7 nicotinic acetylcholine receptors increases intracellular cAMP levels via activation of AC1 in hippocampal neurons. *Neuropharmacology* **95,** 405–414 (2015).
59. Ghasemi, R., Moosavi, M., Zarifkar, A., Rastegar, K. & Maghsoudi, N. The Interplay of Akt and ERK in Aβ Toxicity and Insulin-Mediated Protection in Primary Hippocampal Cell Culture. *J. Mol. Neurosci.* **57,** 325–334 (2015).
60. Cleary, J. P., Walsh, D. M., Hofmeister, J. J., Shankar, G. M., Kuskowski, M. A., Selkoe, D. J. & Ashe, K. H. Natural oligomers of the amyloid-β protein specifically disrupt cognitive function. *Nat. Neurosci.* **8,** 79–84 (2005).
61. Lynch, M. A. Long-Term Potentiation and Memory. *Physiol. Rev.* **84,** 87–136 (2004).
62. Kumar, A. Long-Term Potentiation at CA3–CA1 Hippocampal Synapses with Special Emphasis on Aging, Disease, and Stress. *Front. Aging Neurosci.* **3,** (2011).
63. Benilova, I., Karran, E. & De Strooper, B. The toxic Aβ oligomer and Alzheimer's disease: an emperor in need of clothes. *Nat. Neurosci.* **15,** 349–357 (2012).

64. Lei, M., Xu, H., Li, Z., Wang, Z., O'Malley, T. T., Zhang, D., Walsh, D. M., Xu, P., Selkoe, D. J. & Li, S. Soluble Aβ Oligomers Impair Hippocampal LTP by Disrupting Glutamatergic/GABAergic Balance. *Neurobiol. Dis.* **85,** 111 (2016).
65. Jung, J. H., An, K., Kwon, O. B., Kim, H. & Kim, J.-H. Pathway-specific alteration of synaptic plasticity in Tg2576 mice. *Mol. Cells* **32,** 197–201 (2011).
66. Ma, T., Hoeffer, C. A., Capetillo-Zarate, E., Yu, F., Wong, H., Lin, M. T., Tampellini, D., Klann, E., Blitzer, R. D. & Gouras, G. K. Dysregulation of the mTOR Pathway Mediates Impairment of Synaptic Plasticity in a Mouse Model of Alzheimer's Disease. *PLoS ONE* **5,** e12845 (2010).
67. Tu, S., Okamoto, S., Lipton, S. A. & Xu, H. Oligomeric Aβ-induced synaptic dysfunction in Alzheimer's disease. *Mol. Neurodegener.* **9,** 48 (2014).
68. Rammes, G., Hasenjäger, A., Sroka-Saidi, K., Deussing, J. M. & Parsons, C. G. Therapeutic significance of NR2B-containing NMDA receptors and mGluR5 metabotropic glutamate receptors in mediating the synaptotoxic effects of β-amyloid oligomers on long-term potentiation (LTP) in murine hippocampal slices. *Neuropharmacology* **60,** 982–990 (2011).
69. Zeng, Y., Zhao, D. & Xie, C.-W. Neurotrophins Enhance CaMKII Activity and Rescue Amyloid-β-Induced Deficits in Hippocampal Synaptic Plasticity. *J. Alzheimers Dis. JAD* **21,** 823–831 (2010).
70. Wang, R., Zhang, Y., Li, J. & Zhang, C. Resveratrol ameliorates spatial learning memory impairment induced by Aβ1–42 in rats. *Neuroscience* **344,** 39–47 (2017).

71. Tao, W., Chen, Q., Zhou, W., Wang, Y., Wang, L. & Zhang, Z. Persistent Inflammation-induced Up-regulation of Brain-derived Neurotrophic Factor (BDNF) Promotes Synaptic Delivery of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor GluA1 Subunits in Descending Pain Modulatory Circuits. *J. Biol. Chem.* **289,** 22196–22204 (2014).
72. Cummings, J. L., Morstorf, T. & Zhong, K. Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimers Res. Ther.* **6,** 37 (2014).
73. Rowan, M. J., Klyubin, I., Wang, Q. & Anwyl, R. Synaptic plasticity disruption by amyloid β protein: modulation by potential Alzheimer's disease modifying therapies. *Biochem. Soc. Trans.* **33,** 563–567 (2005).
74. Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongaarts, A., Ohno, M., Disterhoft, J., Van Eldik, L., Berry, R. & Vassar, R. Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J. Neurosci. Off. J. Soc. Neurosci.* **26,** 10129–10140 (2006).
75. Devi, L. & Ohno, M. Cognitive benefits of memantine in Alzheimer's 5XFAD model mice decline during advanced disease stages. *Pharmacol. Biochem. Behav.* **144,** 60–66 (2016).
76. Ardestani, P. M., Evans, A. K., Yi, B., Nguyen, T., Coutellier, L. & Shamloo, M. Modulation of neuroinflammation and pathology in the 5XFAD mouse model of Alzheimer's disease using a biased and selective beta-1 adrenergic receptor partial agonist. *Neuropharmacology* **116,** 371–386 (2017).

77. Segal-Gavish, H., Danino, O., Barhum, Y., Ben-Zur, T., Shai, E., Varon, D., Offen, D. & Fischer, B. A Multifunctional Biocompatible Drug Candidate is Highly Effective in Delaying Pathological Signs of Alzheimer's Disease in 5XFAD Mice. *J. Alzheimers Dis.* **58,** 389–400 (2017).
78. Corcoran, K. A., Lu, Y., Turner, R. S. & Maren, S. Overexpression of hAPPswe Impairs Rewarded Alternation and Contextual Fear Conditioning in a Transgenic Mouse Model of Alzheimer's Disease. *Learn. Mem.* **9,** 243–252 (2002).
79. Perez-Cruz, C., Nolte, M. W., Gaalen, M. M. van, Rustay, N. R., Termont, A., Tanghe, A., Kirchhoff, F. & Ebert, U. Reduced Spine Density in Specific Regions of CA1 Pyramidal Neurons in Two Transgenic Mouse Models of Alzheimer's Disease. *J. Neurosci.* **31,** 3926–3934 (2011).
80. Antunes, M. & Biala, G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn. Process.* **13,** 93–110 (2012).
81. Ennaceur, A. & Chazot, P. L. Preclinical animal anxiety research – flaws and prejudices. *Pharmacol. Res. Perspect.* **4,** (2016).
82. Troakes, C. & Ingram, C. D. Anxiety behaviour of the male rat on the elevated plus maze: associated regional increase in c-fos mRNA expression and modulation by early maternal separation. *Stress* **12,** 362–369 (2009).
83. Jawhar, S., Trawicka, A., Jenneckens, C., Bayer, T. A. & Wirths, O. Motor deficits, neuron loss, and reduced anxiety coinciding with axonal degeneration and intraneuronal Aβ aggregation in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol. Aging* **33,** 196.e29–40 (2012).

84. Pugazhenthi, S., Wang, M., Pham, S., Sze, C.-I. & Eckman, C. B. Downregulation of CREB expression in Alzheimer's brain and in Aβ-treated rat hippocampal neurons. *Mol. Neurodegener.* **6,** 60 (2011).

85. Satoh, J., Tabunoki, H. & Arima, K. Molecular network analysis suggests aberrant CREB-mediated gene regulation in the Alzheimer disease hippocampus. *Dis. Markers* **27,** 239–252 (2009).

86. Yiu, A. P., Rashid, A. J. & Josselyn, S. A. Increasing CREB function in the CA1 region of dorsal hippocampus rescues the spatial memory deficits in a mouse model of Alzheimer's disease. *Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol.* **36,** 2169–2186 (2011).

87. Choi, H., Kim, H. J., Kim, J., Kim, S., Yang, J., Lee, W., Park, Y., Hyeon, S. J., Lee, D.-S., Ryu, H., Chung, J. & Mook-Jung, I. Increased acetylation of Peroxiredoxin1 by HDAC6 inhibition leads to recovery of Aβ-induced impaired axonal transport. *Mol. Neurodegener.* **12,** 23 (2017).

88. Devi, L. & Ohno, M. Effects of levetiracetam, an antiepileptic drug, on memory impairments associated with aging and Alzheimer's disease in mice. *Neurobiol. Learn. Mem.* **102,** 7–11 (2013).

89. Devi, L., Tang, J. & Ohno, M. Beneficial effects of the β-secretase inhibitor GRL-8234 in 5XFAD Alzheimer's transgenic mice lessen during disease progression. *Curr. Alzheimer Res.* **12,** 13–21 (2015).

90. B6SJL-Tg(APPSwFlLon,PSEN1*M146L*L286V)6799Vas/Mmjax. at <https://www.jax.org/strain/006554>

91. Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G. & Silva, A. J. Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* **79,** 59–68 (1994).
92. Sekeres, M. J., Neve, R. L., Frankland, P. W. & Josselyn, S. A. Dorsal hippocampal CREB is both necessary and sufficient for spatial memory. *Learn. Mem. Cold Spring Harb. N* **17,** 280–283 (2010).